Characterization of Endo-D-Xylanase Activity from Streptomyces Flavogriseus and Streptomyces Wedmorensis

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BY
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CERTIFICATE

This is to certify that the research work embodied in this dissertation entitled "**Characterization of endo-D-xylanase activity from Streptomyces flavogriseus and Streptomyces wedmorensis**" submitted to the Aligarh Muslim University, Aligarh, in partial fulfilment of the requirement for the award of the degree of Master of Philosophy is an original work, unless otherwise stated, carried out by **Simi Ali** under our joint guidance in the Division of Microbial Genetics, Central Drug Research Institute, Lucknow.

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C O N T E N T S

	Page No.
Preface	1 - 3
Introduction & Review of Literature	4 - 46
General Materials and Methods	47 - 56
Results	57 - 66
Discussion	67 - 73
Bibliography	74 - 83

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PREFACE

Dwindling world reserves of fossil fuels have focussed attention on the development of alternate source of energy and chemicals hence biomass has emerged as good alternative. A typical biomass consists of cellulose, hemicellulose and lignin in 4:3:3 ratio. Research and development on lignocellulose bioconversion processes have evolved around two major paths i.e. chemical or enzymatic hydrolysis resulting in product of hydrolysate sugar which can subsequently be used as chemical or converted into energy.

Hemicellulose is a mixture of hexose and pentose sugars with xylan being the most abundant component. Xylan can be hydrolyzed by number of organisms including fungi, bacteria, insects, snails and plants through enzymatic route by synthesizing D-xylanases [E.C. 3.2.1.8] and β-D xylosidases (E.C. 3.2.1.37). Enzymatic hydrolysis can have high product yield with low byproduct formation but suffer from large pretreatment energy requirement, short life of enzyme and high cost of carrying out sterile fermentations for producing the enzyme.

Thus for efficient enzymatic hydrolysis, a strain would be recommended which would utilize xylan in native hemicellulose state. Fungi are found to penetrate and colonize the wood but their large scale cultivation is often difficult because of slow generation time, co-production of highly viscous polymers and

poor oxygen transfer. Besides fungi actinomycetes are also well associated with wood and its colonization of wood in soil and marine environment is reported. Short term culture studies showed that members of genus Streptomyces could rapidly colonize and extensively penetrate wood tissue. Streptomyces seem to closely resemble their fungal counterparts than other cellulose degraders, relative to bacterial anaerobes, Streptomyces produce a large amount of extracellular xylanase activity and offer a potential advantage over fungi in being prokaryote and that genetic knowledge and cloning technology is available, which makes them attractive candidates for genetic manipulation.

High enzyme yielding strains have been isolated in past, by mutagenizing the natural isolates. With advent of recombinant DNA technology, it has been possible to isolate and clone the genes for specific enzymes in multicopy vector, thereby giving a gene dosage effect. Transforming the biomass would permit not only a new source of energy and chemicals but would also permit the exploration of new areas which have been untouched till now.

Before substantial utilization of Streptomyces for hemicellulose conversion can be useful more has to be known about the way they decompose hemicellulose, the nature of enzymes involved and optimization of various steps leading to degradation. Since Streptomyces can be genetically manipulated proper characteri-

zation of strain with respect to enzyme is essential for making a right choice of donor strain for isolation of genes coding for xylanase.

In present work we have characterized two strains viz. S.flavogriseus and S.wedmorensis in terms of optimum temperature, pH, thermal inactivation, substrate stabilization, effect of inhibitors and product inhibition. S.wedmorensis is found to code for xylanase which is stable even in alkaline range of pH and temperature $50^{o}C$. These data will aid in our overall objective of enhanced enzyme production by genetic manipulation.

REVIEW OF LITERATURE

Over the past quarter century there has been extensive research into the hydrolysis and fermentation of cellulose. This has included not only the direct production of glucose by acidic and enzymatic hydrolysis and related efforts to improve cellulose yields from promising microbial species but also the production of solvents, fuels and single cell protein by fermentation. By contrast relatively litle has been done with the hemicelluloses that are associated with cellulose in its natural lignocellulose form. The enzymatic breakdown of lignocelluloses using cellulases alone is unlikely to be cost effective without the concurrent use of xylanases. Successful conversion of these materials which are often found in amounts approaching that of cellulose itself could in many cases determine the economic success of the cellulose conversion process under development.

Xylan is the most abundant component of hemicellulose and is found in hard woods, straw, corncobs and grasses in abundance. Xylan is a β 1,4 glycosidic linked polymer of D-xylose, the anhydro-D-xylose units are generally linked β (1→4) ranging from 80-200 units but characteristic variation in structure such as the material is usually branched with these branches containing varying amounts of L-arabinofuranosyl and D-xylo, D-gluco, D-galacto-pyranosyl residue along with acetate and uronic acids (Timell, 1964; Aspinall and McKay, 1958; Timell, 1967; Dekker and Richards, 1976). Xylans possessing (1→3) xylopyranoside bonds are found in marine algae (Timell, 1964).

The structure and chemical heterogeneity has made research into xylan utilization a difficult challenge, while acidic hydrolysis of xylan is easier (Kusakabe et al., 1975) than that of cellulose, enzymatic hydrolysis has also drawn interest. Attempts have been made to obtain xylose from xylan by combination of both.

SOURCE OF ENZYME

The term hemicellulose was introduced by Schulze and hydrolysis of xylan by microorganisms was probably first examined by Hoppe-Seyler (1889), while Sorenson in 1953 gave first evidence that certain bacteria act on xylan to produce xylooligosaccharides and D-xylose. In nature a number of microorganisms including Fungi and bacteria, insects, snails and plants readily hydrolyze the polysaccharide xylan by synthesizing D-xylanases (E.C.3.2.1.8) and D-xylosidases (E.C. 3.2.1.37).

Prokaryotes producing xylanases mainly belong to species within the genera Streptomyces and Bacillus, strains of xylan fermenting have also been reported from the sheep and bovine rumen (Howard et al., 1960; Dehority, 1973).

Fungi from both Basidomycetes and Ascomycetes have been reported to readily hydrolyze this polysaccharide practically all the studies of β-D-xylanases and β -D-xylosidase have been carried out on eukaryotic microorganisms belonging to genera Aspergillus, Fusarium, Cryptococcus, Schizophyllum and Trichoderma which are good producers of extracellular β -D-xylanases (Gorbacheva

and Rodionova, 1977; Gascoigne and Gascoigne, 1960; Biely, 1980; Paice et al., 1978 and Robinson, 1984).

XYLAN DEGRADATION PATHWAY

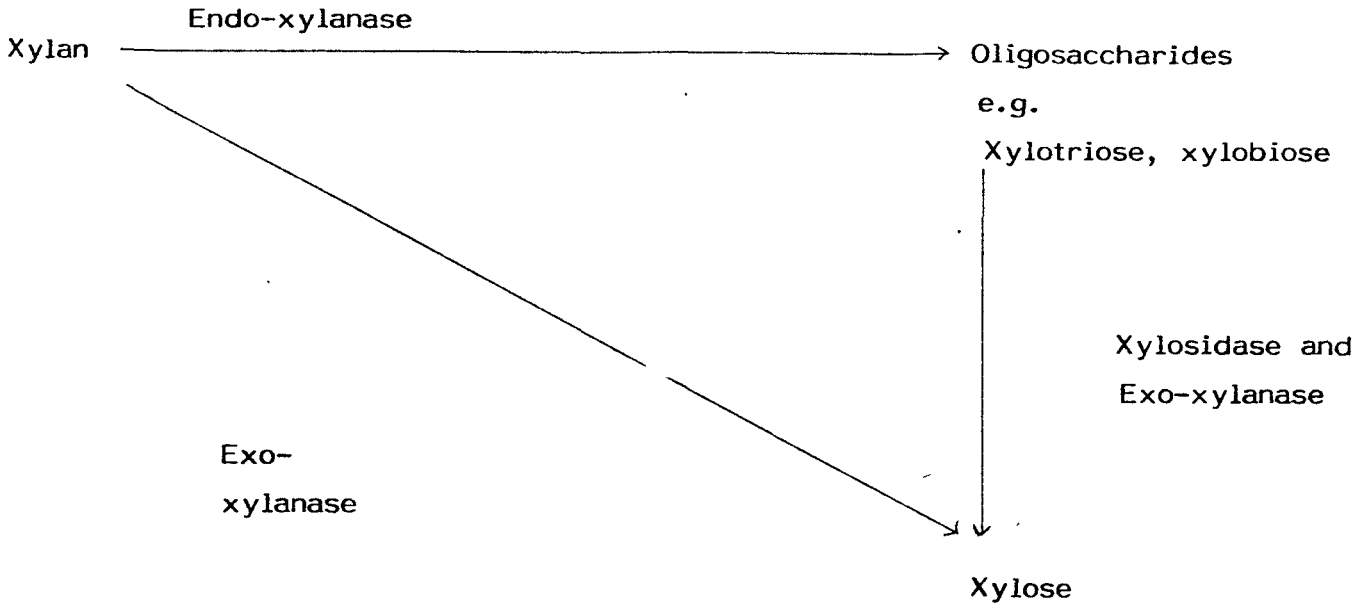
Xylan can be enzymatically hydrolyzed to xylose which can be converted to economically valuable products such as xylulose, xylitol and ethanol. Several types of enzymes are involved in this process, chief among these are hydrolases of three classes: (i) Exo-β-xylanases - which attack xylan and xylooligosaccharides at the non-reducing end also yielding xylose.

(ii) Endo-β-xylanases (1,4-β-D-xylan, xylanohydrolase E.C.3.2.1.8)- This enzyme catalyzes the hydrolysis of β 1,4 xylan at random more readily the xylosidic linkages near the middle of the chain (Nakanishi et al., 1976) and longer oligosaccharides at various points throughout the molecules.

(iii) β -xylosidases (E.C. 3.2.1.37) - It is another important enzyme in xylan degradation pathway and acts on wide range of xylosaccharides by step-wise removal of single xylose units. Xylosidase operates like a typical glycosidase hydrolyzing xylotriose, xylobiose and higher oligomers but not xylan. Thus oligosaccharides produced by endo-β D-xylanases are subjected to xylosidase action to give xylose as final product.

MECHANISM OF XYLAN HYDROLYSIS

Two groups have investigated mechanism of xylan hydrolysis thoroughly.



XYLAN DEGRADATION PATHWAY

Coumtat and Joseleau (1981) found that an endoxylanase from Sporotrichum dimorphosporum attacked redwood xylan to give large amounts of acidic sugars xylobiose; xylose and α-L-arbinofuranosyl-[1 → 3] xylobiose. A less common product had L-arabinose linked in the same manner at the non-reducing end of xylotriose. Non-dialyzable products high in arabinose, rhamnose, galactose and uronic acids accumulated during hydrolysis. Most of this dialyzable acidic sugars contained 4-O-methyl glucuronic acid attached to the O-2 of the non-reducing terminal xylosyl residue of xylotriose and xylotetraose. Most of the arabinose that was found among the dialyzable sugars was part of the acidic component. From this reaction pattern it appeared that xylanase required at least a xylotriosyl group for hydrolysis, the xylosyl residue at the non-reducing end unsubstituted at O-2, the central xylosyl residue unsubstituted at O-2 and O-3 and the xylosyl residue of the reducing end branched or unbranched. Cleavage occurred at the β (1→4) bond linking the central and reducing end xylosyl residues.

Coumtat (1983) separated xylanases from Sporotrichum dimorphosporum by DEAE-sephadex and preparative isoelectrofocussing. Two enzymes with PI 4.4 and 4.7 were brought to homogeneity. All enzymes tested were endoxylanases. One was inhibited by xylobiose while other was activated by it.

The second group investigating xylanase mechanisms was that of Biely, 1980. They purified an endoxylanase from the yeast

Cryptococcus albidus that had no activity on xylobiose, CMC or crystalline or treated cellulose, but some on phenyl-β-D-xylopyranoside. However, xylobiose not xylose was the predominant product from the later along with a phenolic ring (Biely et al., 1980). Increase of activity was sigmoidal with increase of substrate and phenyl β-D-xylobiose and phenyl β-D-xylotriose were intermediates. In addition a lag in the reaction of the xylanases with phenyl β-D-xylopyranoside could be eliminated if xylooligosaccharides were added. All of this indicated that the first step of aryl-xyloside hydrolysis was the transfer of the substrate to xylo-oligosaccharide acceptor (Biely, 1980). Measurement of maximum rates and Michaelis constant for linear xylooligosaccharides or varying lengths and bond cleavage frequencies using reducing end labelled substrates indicated that the two subsites with strong affinities for the substrate were located two positions to either side of cleavage point (Biely et al., 1981). Negative affinities further to the reducing side ensured that the favoured labelled product from substrate larger than xylotriose was xylobiose. However, when xylooligosaccharide concentration increased bond cleavage frequency changed to favour production of large labelled products (Biely et al., 1981). This was caused by adsorption at the active centre of two substrate molecules and by significant transfer ability of the enzymes.

Xylanases from Aspergillus niger appeared to have a carboxyl group in the active centre as with Cryptococcus albidus

enzyme, bond cleavage frequencies changed with increasing substrate came to favour longer labelled products (Vrsańska et al., 1982). They found, however, no attack in phenyl-β-D-xylopyranoside nor did it serve as an acceptor high concentration of short substrate caused the molecule being cleaved to adhere to the enzyme so that the cleavage was further removed from reducing end, with another substrate molecule also absorbed to the remaining subsites. This behaviour was accompanied by formation of products larger than the substrate. Thus Aspergillus niger xylanase appears to have seven subsites with the cleavage point between 3rd and 4th from the reducing end.

Recent literature has confirmed what was noted earlier, many organisms produce more than one endoxylanase and endoxylanases from the same or different organisms have quite different substrate specificities, both in the shortest xylooligosaccharides they can attack and in substrate not containing xylose that are susceptible to hydrolysis. Different products are also formed with same endoxylanase producing mainly small molecules because they have few subsites or because the cleavage point is towards one end of subsite array, others have more subsites and can attack only longer substrate. If cleavage point is towards the middle of the array predominant product will be longer. As it is shown in work of Cryptococcus albidus (Vrsanska et al., 1982) and Aspergillus niger (Gorbacheva and Rodionova, 1977). Transferase activity is possible in endoxylanase and it is accompanied by

a change in the nature of hydrolysis products. From Bacillus species, a thermostable xylanase has been reported with significant activity on cellulose or CMC which produced xylobiose through xylotetrose with arabinosyl xylotriose and same xylose but no arabinose from xylan (Uchino and Nakane, 1981).

Endoxylanase purified from Bacillus pumilus first produced large oligosaccharides and then a mixture of xylobiose through xylopentaose from xylan. No xylose or arabinose was formed the enzyme had transferase activity as xylotetraose and xylobiose were formed from xylotriose and a mixture of xylose to xylopentaose was obtained from xylotetraose. The xylanases have been purified to homogeneity from Bacillus subtilis (Bernier, 1983). The main product from xylan was xylobiose with traces of xylose and xylotriose.

Xylanases from a number of fungi have ben investigated in last few years (Takahashi and Kutsumi, 1979) and purified to crystallinity. Endoxylanase from Gliocladium virens that is highly active on xylan yields mainly, xylobiose with some xylose, xylotriose, was the smallest oligosaccharide attacked by this endoxylanase to give largely xylobiose which suggested that transfer activity must have been present. There was weak activity on CMC, cellulose and starch, of three xylanases from Talaromyces byssachamydoides, One produced a mixture of glucose arabinose and a larger oligosaccharides from xylan while the other two yielded mainly xylose and xylobiose (Yoshioka et al.,1981).

A xylanase from Trichosporon cutaneum initially yielded xylobiose, xylotriose and xylotetraose from xylan and eventually xylose, xylobiose and xylotriose (Stuttzen and Sahm, 1982). No arabinose was found.

GROWTH CONDITIONS

For production of desired enzyme the appropriate micro-organism is cultivated in suitable medium at an appropriate environmental conditions of pH, temperature, oxygen-tension etc., for a definite time period. Culture conditions exert significant influence on physiology and metabolism of microbes for their synthesizing capacity. In order to maximize production of a particular protein, manipulation of culture conditions is very essential. The culture requirement and conditions vary depending on the nature of organism which produces the xylanolytic enzymes. The cultivation conditions of some important xylanase producing microorganisms and their yields are listed in Table I.

It is important to note that culture growth period is an important factor in providing an optimum yield and productivity of enzyme. Thus proper assessment of cultivation time and the conditions of harvesting the cell on a large scale is essential to get high production.

EXTRACTION AND PURIFICATION

After cultivation of the desired strain for a definite time period the extracellular enzyme fraction is collected by pelleting

the cells at 10,000 g for 7 minutes. Xylanase activity is assumed to be extracellular as known for polysaccharide degrading enzymes.

β-xylosidase activity is usually cell bound (Roncero, 1983) hence for intracellular fraction preparation, extraction steps include (a) cell lysis (b) removal of cell debris to give clear cell extract.

Following techniques have been employed for cell lysis

(1) Osmotic shock of Spheroplast prepared by lysozyme.

(2) Explosion decompression in French press.

(3) Mechanical grinding with alumina or glass beads.

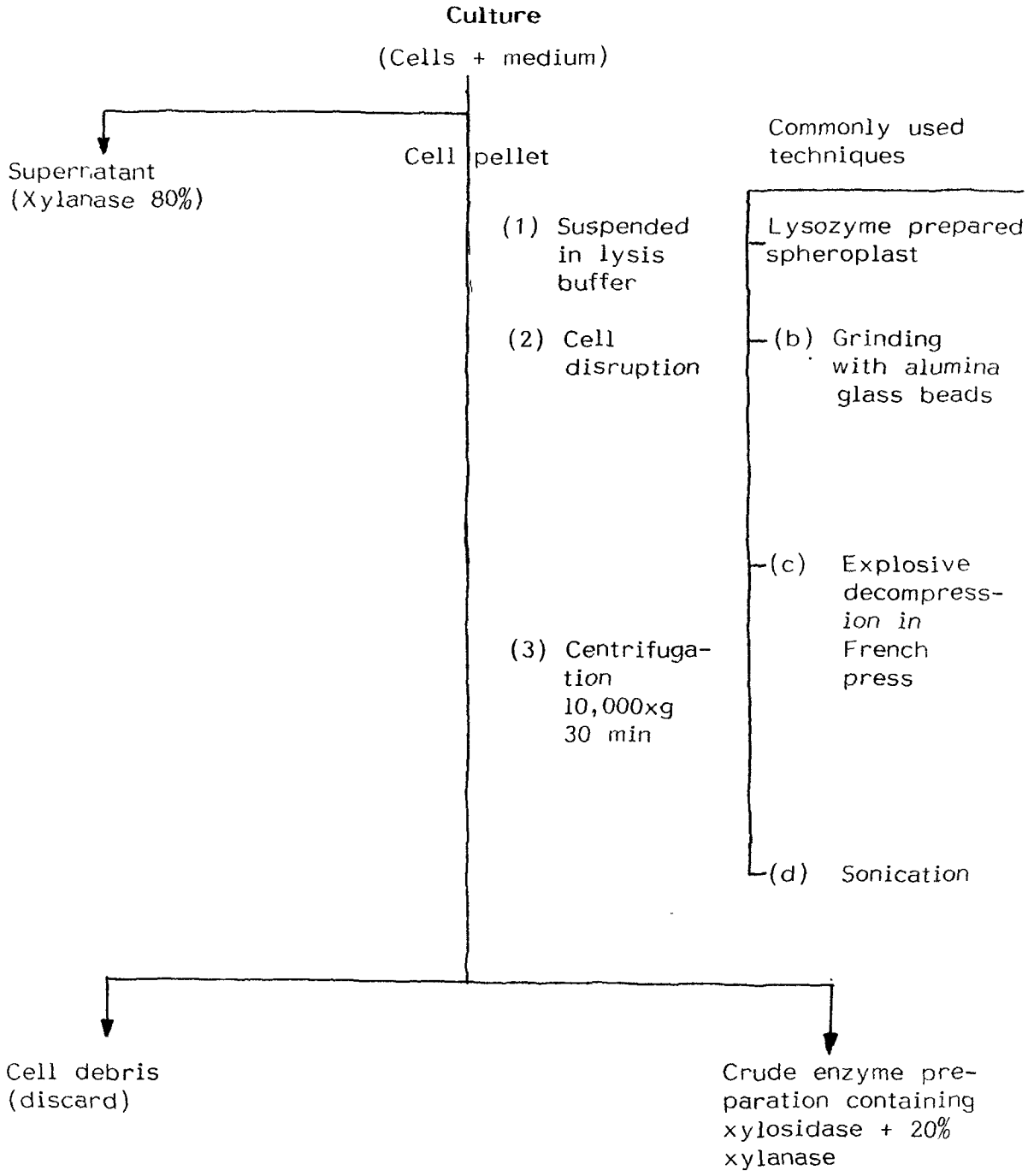
(4) Sonication.

(5) Freezing and thawing

Sonication is most widely used. Temperature during sonication was never permitted to go beyond $10^{o}C$. The degree of cell lysis is monitored either by microscopic observations or sometimes by absorbance at 280 nm (Greene et al., 1978). After sonication cell debris and subcellular materials are removed by centrifugation at 10,000g for 30 min, the supernatant is used as intracellular enzyme fraction. It has been concluded that β -xylosidase is cell associated and probably membrane associated (Deleyn and Claeyssen, 1978).

The extracellular culture filtrate and the cell extract have been used as source of endoxylanase and xylosidase for further purification. Typical steps followed in extraction procedure are illustated in Table II.

Purification of enzyme includes protein precipitation using specific reagents like ammonium sulphate, polymin P etc., dialysis



STEPS INVOLVED IN EXTRACTION OF INTRACELLULAR FRACTION OF ENZYME

Table I: Production media culture conditions of few xylanases.

Organism	Production media composition		Cultivation condition		Xylanase enzyme yield	Reference
Bacillus sp. W_1	Xylan	10 gm	Temp.	45°C	88.5 IU/ml	Okazaki et al.(1984)
	Tryptone	10 gm	pH	10		
	Yeast extract	5 gm	Time	48 hrs		
	NaCl	5 gm				
	Distilled water	1 litre				
Bacillus pumilus	Xylan	25 gm	Temp	30°C	16.0 IU/mg	Panbangred et al. (1983)
	KH_2PO_4	2 gm	pH	6.5		
	NH_4NO_3	2 gm	Time	48 hrs		
	$MgSO_4.7H_2O$	2 gm				
	Yeast extract (DIFCO)	0.25 gm				
	Distilled water	1 litre				
Bacillus circulans WL-12	Yeast nitrogen base	6.7 gm	Temp	30°C	121.6 n mol of R.S. $min^{-1}ml^{-1}$	Esterban (1982)
	Xylan	5 gm	pH	6.5		
	Distilled water	1 litre	Agitation rate	250 rpm		
	pH	6.5				

[contd.....]

[Table 1: contd...]

Bacillus subtilis	Xylan	10 gm	Temp	$30^{o}C$	3420 I.U.	Bernier et al(1983)
	KH_2PO_4	2 gm	pH	6.5	Sp. activity	
	$MgSO_4 7H_2O$	1 gm	Time	72 hrs	38 : I.U./mg	
	$FeSO_4 7H_2O$	0.05gm	Agitation	200 rpm		
	$MnSO_4$	0.05 gm				
	$CaCl_2 2H_2O$	0.1 gm				
	Urea	2.5 gm				
	Cellobiose	1.0 gm				
	Tryptophan	0.04 gm				
	Thiamine	0.001 gm				
	Distilled water	1 litre				
Clostridium acetobutylicum ATCC 824	OAT spelt Xylan	15 gm	Time	18 hrs	Extracellular:	Lee et al. (1987)
	Xylose	5 gm	pH	6	115.1 nmol of R.S. $min^{-1}ml^{-1}$	
	In Chemostat		Dilution rate	0.05 h^{-1}	Cell bound: 65.7 nmol of R.S. $min^{-1}ml^{-1}$	

[Contd.....]

[Table 1: contd...]

Cellulomonas flavigena NIAB 441	Cellulose Hemicellulose Maintenance Medium (Choudhary et al. 1980) Distilled water	10 gm 1 litre	Temp pH Time	30°C 7.3 3 days	16.0 I.U/ml	Rajoka and Malik (1984)
Chiana NcL 82-5-1	Xylan Yeast extract Distilled water	30 gm 10 gm 1 litre	Temp pH Time Agitation	28°C 7.8 72 hrs 220 rpm	28.0	Srinivasan and Vartak (1984)
Penicillium funiculosum	Xylan	1%	Temp Time Agitation	28°C 10 days 220 rpm	16.0	Mishra et al (1985)
Cellulomonas MUTANT SI-17	Xylan Tryptone Yeast extract Sodium chloride Distilled water	5 gm 10 gm 5 gm 5 gm 1 litre	Temp Time	28°C 48 hrs	16.7 I.U/ml	Peiris and Richard (1982)

[contd.....]

[Table 1: contd....]

Fusarium roseum	KCl	0.5 gm	Temp	30°C		Gascoigne and Gascoigne (1960)
	$MgSO_4.7H_2O$	0.5 gm	pH	6.7		
	$(NH_4)_2HPO_4$	2.5 gm	Time	13 days		
	NaH_2PO_4	0.5 gm	Agitation	200 Oscilla-		
	$CaCl_2.H_2O$	0.01 gm		tions/min		
	$FeSO_4 7H_2O$	0.01 gm				
	$ZnSO_4.7H_2O$	0.002 gm				
	pH	6				
	Distilled water	1 litre				
	Xylan	1%				
Schizophyllum commune	Black Spruce Saw dust	40 gm	Temp	30°C	1 gm crude enz/ litre	Paice et al. (1978)
	Peptone	2.5 gm	pH	3.9		
	$Ca(NO_3)_2.4H_2O$	7.2 gm	Time	9 days		
	KH_2PO_4	0.5 gm	Agitation	200 rpm		
	$MgSO_4.7H_2O$	0.5 gm	rate			
	Distilled water	1 litre				

[contd....]

[Table 1: contd...]

Streptomyces lividans 1326	Xylan	10 gm	Temp	34°C	50 I.U/ml	Morosoli et al.(1986)
	$(NH_4)_2SO_4$	1.4 gm	pH			
	K_2HPO_4	2.5 gm	Time	48 hrs		
	KH_2PO_4	1.0 gm	Agitation	250 rpm		
	Yeast extract (Difco)	2 gm				
	Protease peptone	1 gm				
	$MgSO_4.7H_2O$	0.3 gm				
	$CaCl_2.2H_2O$	0.3 gm				
	Tween-80	2 ml				
	Trace metal solution	1 ml				
	Distilled water	1 litre				
	Trace Metal Solution					
	$COCl_2.6H_2O$	0.2 gm				
	$FeSO_4.7H_2O$	0.5 gm				
	$MnSO_4.H_2O$	0.16 gm				
	$ZnSO_4.7H_2O$	0.14 gm				
	Distilled water	100 ml				
	pH	3.0				

[contd...]

[Table 1: contd...]

Streptomyces sp. No. 3137	Methy-β xyloside	0.3 gm	Temp	30°C	37.5 I.U	Marui et al.(1985)
	KH_2PO_4	0.2 gm	Time	40 hr		
	$MgSO_4.7H_2O$	0.01 gm	Agitation	120 strokes/		
	Distilled water	1 litre		min		
Streptomyces flavogriseus	K_2HPO_4	2 gm	Temp	30°C	46 I.U	Ishaque and Kluepfel (1981)
	KH_2PO_4	1.5 gm	pH	7		
	Yeast extract (Difco)	2.0 gm	Time	48 hrs		
			Agitation	240 rpm		
	Protease peptone	1 gm				
	Tween-80	2 ml				
	Trace metal solution	1 ml				
	(Mandel a-Reese 1975)					
	Xylan	10 gm				
	Distilled water	1 litre				
Streptomyces sp. KT-23	Rice straw	0.5 gm	Temp	30°C	125 units/mg	Nakajima et al. (1984)
	Arabino xylan		pH	7	of protein	
	KH_2PO_4	2.0 gm	Time	48 hrs	21,400 units	
	K_2HPO_4	3.2 gm	Agitation	130 Oscill-	(Total activity)	
	$MgSO_4.7H_2O$	0.5 gm		ation/min		
	Yeast extract	0.5 gm				
	$FeSO_4.7H_2O$	0.2 gm				
	Dist. water	1 litre				[contd...]

[Table 1: contd....]

Sclerotium rolfii	Nm	2	Temp	30°C	110 I.U./ml	Sadana *et al.*(1980)
U.V.8	KH_2PO_4	2.0 gm	pH	7		
	$(NH_4)_2HPO_4$	2.0 gm	Time	14 days		
	Urea	0.3 gm	Agitation	150 rpm		
	$MgSO_4.7H_2O$	0.3 gm				
	$CaCl_2.2H_2O$	0.3 gm				
	Protease peptone	0.25 gm				
	Yeast extract	0.1 gm				
	Tween-80	0.3 gm				
	Cellulose	12.3-30.0 gm				
	Distilled water	1 litre				
Trichoderma reesei	Xylan	1%	Fermentor	7L	130 I.U/ml	Robison (1984)
C-30	Natuk media		Temp	26°C		
			Time	9 days		
			pH	3.0		
Thielavia terrestris	Solkafloc	1%	Fermentor	14L	18.8 I.U/ml	Merchant *et al.*
ATCC 26017			Temp	48°C		(1988).
			Time	18 hrs		
			pH	4.0		
			Agitation	100 rpm		

Table No. II: Major process steps used in purification of new xylanases

Organism	Sequence of steps during purification	Reference
Bacillus circulans	CRUDE PREPARATION ↓ DEAE Biogel A ↓ Fraction I has xylanase + xylosidase activity ↓ Biogel A ↓ eluted with NaCl gradient Fraction concentrated by ultrafiltration ↓ Sephadex S-300 column ↓ Two enzymes not separated ↓ Isoelectric focussing column ↓ Xylanase pI-4.45 / Xylosidase pI - 4.7	Estesban et al. (1982)
Bacillus subtilis	CRUDE PREPARATION ↓ Ethanol precipitation ↓ SP - Sephadex ↓ S-200 ↓ Purified enzyme (Endoxylanase)	Bernier et al. (1983)

Bacillus pumillus — Panbangred *et al.* (1983)

Culture fluid
↓
$(NH_4)_2SO_4$ fractionation
↓
DEAE Sephadex column chromatography
↓
CM-Sephadex column chromatography
↓
Purified enzyme (Endoxylanase)

Clostridium acetobutylicum — Lee (1987)

Crude preparation
↓
CM Sepharose

Peak A
Failed to bind to gel had xylosidase, arabinofuranosidase, xylanase
↓
Hydroxylapatite chromatography

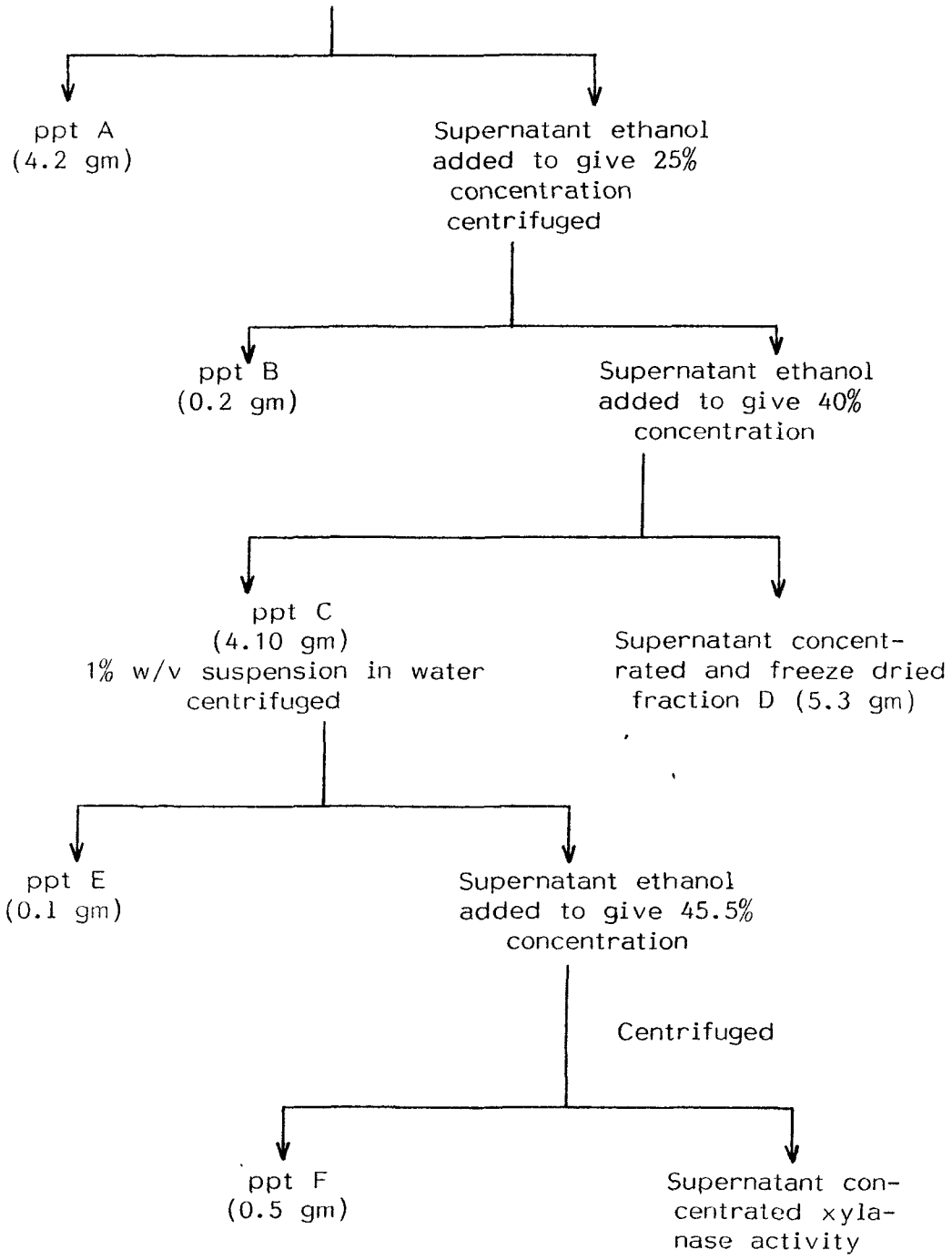
Peak B
Eluted in NaCl gradient
↓
Hydroxylapatite column
↓
Xylanase B

↓
Hydrophobic chromatography
(Xylanase separated from arabinofuranosidase which binds irreversibly to gel)

Major
↓
Xylanase eluted with 1% Triton X-100
↓
Xylanase freed from detergent by chromatography and hydroxylapatite
↓
Resulting enzyme chromatography on Biogel P-150
↓
Xylanase A

Minor
25% of peak A xylanase

Fusarium roseum	Fractionation of culture medium dialysed solution freeze dried (14 gm) shaken with water and centrifuged	Gascoigne and Gascoigne (1960)



against suitable buffers and several column chromatographic steps involving gel filtration, ion exchange and affinity chromatography.

LOCALIZATION AND NATURE

Xylanase is largely found in the extracellular fraction of culture whereas β -xylosidase is mainly detected in the intracellular preparations. Xylanase is an adaptive or inducible enzyme produced only when grown in medium containing xylan or related polysaccharide. Xylosidase is also an inducible enzyme but it is produced not only when xylobiose is substrate but also during growth on polysaccharides this enzyme could attack oligosaccharides higher than hexaose and it is infact able to release xylose from mixed oligosaccharides but does not act on xylan.

The hemicellulases of fungi occur both intra and extracellularly although it has been shown (Bose and Sarkar, 1937) that some fungi produce more in surroundings than within the cells. It is seen in Streptomyces flavogriseus (Ishaque and Kluepfel, 1981) that considerable amount of xylanase was produced when grown on xylan containing medium, comparatively lower yields of this enzyme were obtained when avicel served as main carbon source. β-xylosidase was synthesized intracellularly and appeared less dependent on fermentation substrate, it was produced uniformly on all substrates reaching optimal level at 72 hr. An accelerated production occurred on xylan containing substrate suggesting a certain initial induction of enzyme. This contrasts with some reports concerning β -D-xylanases in eukaryotic organisms (Manners and Mitchell,

1967) have demonstrated the constitutive nature of the enzymes which are synthesized even if glucose is used as carbon source. Xylanase was reported to be formed constitutively in Clostridium stercorarium (Berenger et al., 1985).

At the same time the fact that xylan the natural substrate, is the best carbohydrate for β-D-xylanase production is in agreement with the results published by Nakanishi et al. (1970). It was reported that xylan is the best inducer of β-D-xylanase activity exhibiting higher induction power than xylooligosaccharides.

In case of Streptomyces sp. 3137 (Marui et al., 1985) this organism did not produce xylanase when grown in medium containing glucose as sole carbon source. However, addition of xylan to the medium stimulated xylanase production which was dependent on concentration of xylan in medium. The addition of nitrogenous substances such as peptone when used together with xylan improved xylanase production.

Xylanases are generally quite small proteins ranging from 15,000 to 30,000 dalton although higher molecular weight xylanases have occasionally been isolated. Xylanases appear to represent an isoenzyme system. Three types of xylanases induced by methyl-β-xyloside have been purified from Streptomyces sp. (Marui et al., 1985), Bacillus circulans secretes 2 endo 1,4-β-D-xylanase (Okazaki et al., 1984), Schizophyllum commune produces several xylanases (Paice et al., 1978), three endo 1,4 xylanases have been

isolated from Aspergillus niger (Gorbachova and Rodionova, 1977), Clostridium acetobutylicum (Lee et al., 1987) produces 2 endoxylanases. These are the examples of some xylanases which represent the isozyme system.

Endoxylanases with activity against cellulose are found among prokaryotes e.g. Bacillus acidocaldarius (Uchino and Nakane, 1981) and in eukaryotes e.g. Trichoderma reesei (Baiely, 1985).

The multiplicity of enzymes in microorganisms could arise from post-translational processing such as proteolysis or glycosylation, no antigenic similarity suggests that they are encoded by two separated genes (Lee et al.,' 1987).

Berenger et al (1985) showed that catabolic repression of xylanase synthesis occurred when glucose and other readily metabolizable substrates were added during growth. The results show that xylanase biosynthesis stopped when glucose, xylose, lactose and cellobiose were added during growth on cellulose and resumed after depletion of these sugars. This repression is also shown to exist in other cellulose degrading bacteria (Stutzenberger, 1971).

Endoxylanases with activity against cellulose have higher specific activity on cellulose which can be attributed either to absence of some repression on cellulose cultures or to presence of some inhibitory compounds in xylan like high concentration of xylose or xylodextrins produced in cultures by hydrolysis of soluble-

xylan, larch (Xylan, USA) has been reported to contain polyphenol residue this could be the other possibility for catabolic repression.

ASSAY OF XYLANASE

Assay method: Enzyme assays were carried out by incubating the enzyme sample with substrate prepared in suitable buffer for 30 min. The reducing sugar released from substrate was either measured according to Miller et al. (1960) or by Somogyi (1952) (as indicated by Ashwel, 1957) referring the reading to xylose as the standard. n-nitrophenol released from p-nitrophenyl β-D-xylopyranoside (PNPX) is evaluated spectrophotometrically (Notario et al., 1976) for xylosidase assay.

UNIT DEFINITION

A conventional unit to define the activity of enzyme is the amount of enzyme that catalyzes the release of umole of reducing sugar as xylose from substrate per millilitre per minute at the optimum temperature.

When testing β-xylosidase activity, the enzyme unit corresponded to the amount of the enzyme, that released umole of p-nitrophenol from PNPX under the same conditions.

PHYSICO-CHEMICAL PROPERTIES

Xylanase enzymes are not so well characterized in terms of physico-chemical properties this is because more attention has been diverted towards their application rather than enzymology. Efforts have, however, been made to explain the structure and

mechanism of action of few xylanases; such as xylanases from Bacillus pumillus, Streptomyces lividans etc., enzyme kinetics and properties like isoelectric point (pI), michaelis mentenconstant (Km), molar extinction coefficient and aminoacid composition are available for few enzymes only. Some of the common physico-chemical properties such as pH and temperature optima in relation to activity and stability and molecular weight for some xylanases are summarised in Table No. IV.

(a) pH and temperature optima and stability

Optimum pH for activity of most of the xylanases is slightly towards acidic side around 5-7 with a maximum value of 3.3-4 for Penicillium wortmani (Deleyen and Claeyssens, 1978) and minimum 7, several xylanases are found to be stable over a wide range of pH e.g. Aspergillus niger (Garbachova and Radionova, 1977). Alkalophilic Bacillus sp. strain C-125 is reported to produce two types of xylanases one having an optimum pH 7.0 and other has a very broad pH range from 5-11 (Honda et al., 1985). Xylanase enzymes of mesophilic origin have a temperature optima ranging from 40 to 65^{o}C while that of thermophilic origin have a temperature optima 80^{o}C obtained from Bacillus stearothermophilus (Sandhu and Kennedy, 1984) another e.g. of thermostable xylanase with high degree of xylan degradation is Clostridium stercorarium.

(b) Km and pI

The values of pI have been reported for xylanases ranging from 4.45-10.26, high isoelectric point is found for several β-xyla-

nases e.g. Trichoderma pseudokoningii has pI value of 9.6 (Baker et al., 1977), for Streptomyces sp. 3137 (Marui, 1985) pI is reported to be 10.26. Km value for some xylanases is summarized in Table No. 4.

(c) Amino acid composition and molecular weight

Xylanases are generally quite small proteins ranging from 15000-30,000 daltons although higher molecular weight xylanases have occasionally been isolated (Gorbachova and Radionova, 1977) isolated an endo β 1,4 xylanase of molecular weight 25,000 dalton from Aspergillus niger, Paice et al. (1978) reported an endo β -D-xylanase from Schizophyllum commune which has molecular weight of 15,000-22,000, whereas Esteban et al. (1982) reported an endo β-D-xylanase from Bacillus circulans W1-12 with molecular wieght 85,000 daltons which is very close to molecular weight reported for β -D-xylosidase. Deleyn et al. (1978) reported a value of 100,000 dalton for β -D-xylosidase isolated from Penicillium wortmanni.

Several xylanases have been characterized in terms of their amino acid composition e.g. xylanase from Bacillus pumillus contains 18 amino acids (Table III) with aspartic acid and glycine contributing the highest fraction and half cysteine contributing the minimum (Panbangred et al., 1983). Nakajima (1984) described amino acid composition for xylanase isolated from Streptomyces sp. KT-23. Amino acid composition of few xylanases are listed in Table III.

Table III: Amino acid composition of Bacillus pumillus.

(Panbangred et al., 1983).

Amino acid	No. of residues per molecule
Tryptophan	14
Lysine	8
Histidine	4
Arginine	7
Aspartic acid	21
Threonine	16
Serine	17
Glutamic acid	13
Proline	7
Glycine	21
Alanine	13
Half cystine	1
Valine	7
Methionine	4
Isoleunine	8
Leunine	8
Tyrosine	9
Phenyl alanine	7
Total amino acid	185)

Amino acid composition of Bacillus subtilis

(Bernier et al., 1983)

Amino acid	No. of residues	Closest range
Aspartic acid	30.85	31
Cystine	4.18	4
Threonine	25.79	26
Serine	34.63	35
Glutamic acid	17.83	18
Proline	16.70	17
Glycine	53.77	54
Alanine	22.11	22
Valine	13.81	14
Isoleunine	9.13	9
Leunine	10.00	10
Tyrosine	12.58	13
Phenylalanine	3.16	3
Histidine	5.93	6
Tysine	14.36	14
Arginine	9.54	10
Methionine	3.14	3

Amino acid composition of Schiphyllum commune

(Paice et al., 1978)

Amino acid	No. of residues	Closest integer
Aspartic acid	31.58	32
Threonine	29.68	30
Serine	37.52	38
Glutamic acid	19.03	19
Proline	13.74	14
Glycine	47.00	47
Alanine	20.92	21
Valine	14.40	14
Methionine	1.44	2
Isoleucine	11.60	12
Leucine	11.00	11
Tyrosine	23.02	23
Phenylalanine	4.96	5
Histidine	4.14	4
Lysine	7.12	7
Arginine	5.81	6
Half cystine	3.95	4
Tryptophan	8.50	9

Amino acid composition of Streptomyces KT-23

(Nakajima et al., 1984)

Amino acid	umol/mg
Aspartic acid	0.773
Threonine	0.436
Serine	1.425
Glutamic acid	1.214
Glycine	1.327
Alanine	0.734
Half cystine	0.049
Valine	0.389
Isoleucine	0.224
Leucine	0.352
Tyrosine	0.097
Phenylalanine	0.166
Lysine	0.253
Histidine	0.252
Arginine	0.203
Proline	0.228

Table IV: Physiochemical properties of some purified xylanases.

Organisms	Subunit molecular wt. and number	Km	Vmax	pI	Optimum temp.	Stability pH	Reference
Bacillus circulans WL-12	A - 85000 B - 15000	A - 8 mg/ml B - 4 mg/ml	- -	A - 4.5 B - 9.1	- -	A-5.5-7 B-5.5-7	Okazaki et al.(1984)
Bacillus pumillus	24000	-	-	-	40°C 85% enzyme activity at 40°C for 30 min.	6.5	Panbangred et al.(1983)
Bacillus subtilis	32000	0.16 mg/ml	7.0×10^3 umole $min^{-1} mg^{-1}$	-	50°C	5.0	Bernier et al.(1983)
Clostridium acetobutylium ATCC 824	Xylanase A - 65000 B - 29000	A - 6.0mg/ml B - 6.7mg/ml	A-22.4umol of R.S. min^{-1} B-22.3umol of R.S. min^{-1} mg of protein^{-1}	A - 4.45 B - 8.50	A - 50°C B - 60°C	A-5.0 B-5.5-6.0	Lee (1987)
Schizophyllum commune	33000	8.37 mg/ml	0.443 umol/ min	-	55°C	5.0	Paice et al(1978)

[contd....]

[Table IV: contd...]

Streptomyces lividans 1326	43000	0.78 mg/ml	0.85 umol/ min per mg of enzyme	5.2	60°C	6.0	Morosoli et al.(1986)
Streptomyces sp. 3137	XI-50,000 XIIA-25,000 XIIB-25,680		XI-7.10 XII-10.06 XIIB-10.26	-	60-65°C Stable upto 55°C	XI-5.5-6.5 XIIA-5.0-6.0 XIIB-5.0-6.0	Marui(1985)
Streptomyces sp. K7-23	43000	0.20 mg/ml	6.9	-	55°C	5.5	Nakajima (1984)
Penicillium wortmanni	100,000		5.0	-		3.3-4	Deleyn and Claeyssens (1978)

STABILIZATION OF ENZYME AGAINST THERMAL INACTIVATION

For technological applications enzymes should be stable under operational conditions for a length of time. Enzyme inactivation is caused due to number of factors such as heat, proteases, oxygen, acidic and alkaline pH and denaturing reagents. From the practical standpoint thermal inactivation is by far the most important mode of enzyme inactivation, as the name implies, this type of inactivation takes place at elevated temperature. The question which can be posed is "why do we want to use enzymes at elevated temperatures to begin with? There are several advantages in using an enzyme at higher temperature such as -

(1) The rates of enzymatic (as well as most chemical) reactions are generally accelerated upon heating. Hence the use of high temperatures would be economically advantageous.

(2) High temperatures in enzyme reactions greatly reduce the likelyhood of bacterial contamination. Such contamination can result in various deleterious effects e.g. the release of enzyme degrading proteases, plugging of filters etc. This problem is particularly severe in food processing operations which are carried out above $60^{o}C$.

(3) From the productivity standpoint it is often desirable to dissolve as much substrate as possible. Solubility of most substrates increases with temperature.

Thus these factors necessitate the use of thermostable enzyme.

Mechanism of thermal inactivation

There is still much uncertainty about the mechanism of thermal inactivation of enzymes, however, some pathways appear to be well established. The first crucial step in enzyme inactivation is partial unfolding of the molecule (Kauzmann, 1959; Tanford, 1968; Lapanze, 1978).

Under normal conditions the native catalytically active structure of an enzyme is maintained by a delicate balance of different non-covalent forces, hydrogen bonds, hydrophobic, ionic and Vander Waals interaction etc. (Schulz and Schirmer, 1979; Alber et al., 1987) on increase in temperature all these forces (except for hydrophobic interactions which however are significant only upto $60^{o}C$) diminish and the protein macromolecule unfolds i.e. acquires a less ordered conformation. Because the active centres of enzymes always consist of several amino acid residues brought together only in native three dimensional structure of the enzyme (Anfinsen and Sherager, 1975) such unfolding results in a disassembling of the active centre and hence enzyme inactivation, while unfolding seems to be a universal and general phenomenon in enzyme inactivation the subsequent steps are highly specific for individual enzymes. They can be divided in 2 groups covalent and non-covalent.

It should be stressed, however, that unfolding of proteins is usually reversible (Tanford, 1968) irreversiblity of thermal inactivation of enzymes is brought about by subsequent steps that follow the unfolding of enzymes.

Table V: Thermal stability.

Organism	Temperature	Effect	Reference
Bacillus strain DLG	50°C	6% loss of activity after 2 hrs	Robson and Chambliss (1984)
Bacillus subtilis	60°C	Unstable for longer than 20 min	Bernier et al (1983)
Clostridium acetobutylicum	40°C	Stable for 30 min	Lee et al(1987)
	50°C	Stable for 30 min	
Schizophyllum commune	30°C	90% of activity retained after 65 hrs	Paice et al. (1978)
Streptomyces sp. 3137	4°C	Six months	Marui (1980)
	55°C	Stable on heating for 10 min,	
	60°C	Denatured	
Streptomyces lividans	0°C	Stable	
	25°C	Stable for more than 72 hrs	Morosoli (1986)
	37°C	Slight inactivation	
	4°C	after 24 hrs	
	60°C	Half life 30 min	

Methods of enzyme stabilization

(1) Immobilization of enzymes on solid support

(2) Intramolecular cross linking of enzymes

(3) Selective chemical modification of enzyme

(4) Stabilization by neutral salts

(5) Stabilization by ligands

Thermal stability of some of the enzymes is indicated in Table 5.

GENETICS OF XYLANASE

Roncero (1983) in Bacillus subtilis by reciprocal transformation crosses revealed the existence of two genes controlling xylan utilization which have been designated xyn A and xyn B. Available data indicated that the two genes code for two xylan degrading enzymes existing in the wild type strain as extracellular β-xylanase and a cell associated β-xylosidase.

Genetic and biochemical data are mutually consistent and lead to following conclusion - 2 enzymes, 1, 4 β-xylanase and β-xylosidase are responsible for xylan utilization by Bacillus subtilis. The structural genes coding for these two enzymes are tightly linked and are located in map position 50 min of B.subtilis chromosome. They observed linkage of xyn A and xyn B in Bacillus subtilis chromosome. This clustering is characteristic of functionally related genes in prokaryotic organism. Because of low recombination frequencies observed between two mutations, it appears that they are different alleles of same gene.

Another gene for an extracellular xylanase from Streptomyces sp. No.36 was cloned in S.lividens using a PIJ 702n as a vector plasmid. The smallest DNA fragment encoding the xylanase gene and its possible promoter was found to be 1.04 kb. Sph I - Sac I fragment by subcloning studies. The xylanase gene fragment was transferred into pSK2 series of plasmids and introduced into Streptomyces kasugaensis viz. protoplast. The cloned xylanase gene was expressed in both S.lividens TK 21 and Streptomyces kaugeneis 63 and these clones produced and secreted high yields of xylanase intoculture medium (Iwasaki et al., 1986).

Extracellular production of alkaline xylanase of alkalophilic Bacillus sp. by E.coli carrying pCX 311 has been reported by Honda et al. (1985). The enzyme production reached its maximum in 1/3rd of the cultivation period required by alkalophilic Bacillus sp. strain C-125 furthermore the activity detected in culture broth was higher than that of xylanase produced by alkalophilic Bacillus sp.

Structure and expression of genes coding for xylan degrading enzyme have been reported by Moriyama et al. (1987) from Bacillus pumilus. The complete nucleotide sequence of -xylosidase (xyl B) and its flanking region has been established (Fukusaki et al., 1984). The xylanase gene was 4.6 kbp downstream of the 3' end of xyn B and its DNA sequence is repeated. The xylanase gene from Bacillus polymyxa has been cloned and expressed in E.coli (Yang et al., 1988) after extensive mapping and series of subcloning

into pucl9 A 2.9 kilobase BamHI-EcoRI subfragment was found to code for xylanase activity. Xylanase activity expressed by E.coli harbouring the cloned gene was located primarily in periplasm and corresponded to one of the two distinct xylanase, produced by B.polymyxa. Xylanase expressed by cloned gene occurred in absence of xylan and was reduced by glucose and xylose the xylanase expressed by cloned gene had mol.wt. 48,000 and isoelectric point 4.9. Complete nucleotide sequence of xylanase gene of Bacillus pumilus, alkalophilic Bacillus sp. strain C-125 has been reported (Fukasaki et al., 1984; Hamamoto et al., 1987). A xylanase gene of Bacillus pumilus has been expressed in E.coli and Bacillus subtilis (Panbangred et al., 1985). Xylanases have been cloned in E.coli with the aim of obtaining increased production of the enzyme.

SOCIO-ECONOMIC IMPORTANCE

The need of utilizing renewable resources for meeting mankinds future need of food and fuel focussed attention on the renewable polysaccharide produced in tons notably in tropical regions of world, which have only scanty fossil fuel reserves. That the fossil fuels of present day are but products of earlier geologic age also added optimism towards achieving such an object. Microorganisms able to breakdown lignocellulose have a great economic potential for increasing the utilization of renewable plant biomass as an animal feed and for production of fuels and chemicals by fermentation.

Xylan hydrolysis could be of commercial significance since some industrial processes for e.g.

(1) In pulp and paper industry

(a) For fibre modification such as microfibrillation

(b) Extraction of dissolving grade pulps

(c) Bleaching of kraft pulps

For this application, the enzyme solution should be free of cellulose otherwise fibre damage could occur, so molecular cloning of xylanase and xylosidase genes from suitable strains in E.coli (Honda et al., 1985) will produce high levels of cellulase free enzymes.

(2) Xylose obtained from enzymatically treated xylan would be a feed stock for single cell protein products.

(3) Alternatively fermentation to ethanol is possible either by intermediate formation of xylulose, xylitol or with some yeast by direct fermentation (Jeffries, 1981).

Thermophilic bacteria are of particular interest in this respect both in their own right and as a source of desirable gene encoding highly active thermostable protein for genetic construction in mesophiles, the stability of enzyme at high temperature is desirable as in large-scale enzymatic hydrolysis, the temperature usually tends to shoot up some 100 species of bacteria and fungi are known to produce xylanase but comparatively little information is available regarding the biochemical and genetic regulation of these enzymes. At present a number of questions remain unanswered.

(1) Are distinct and different genes present for each of many forms of xylanases and is their expression co-ordinately controlled?

(2) Is the multiplicity of gene a consequence of gene reiteration?

In recent years many advances have been made in developing acid hydrolysis process for xylan hydrolysis. But problems of large energy requirement, high equipment cost, corrosion problem, low product yield and fermentation of mixtures containing toxic or unfermentable byproducts as well as non-specific nature of acid hydrolysis have directed the research efforts towards enzymatic hydrolysis which would be more specific and operative under relatively mild conditions.

Cloning and expression of genes in heterologous host is not only to get better understanding of the regulation of gene but also to construct a high enzyme producing strain by cloning and expressing gene in multicopy vector, with a higher specific activity than observed in natural isolates observed so far. There are several hurdles like -

(1) The strategies of cloning xylanase gene from a prokaryote host cannot rely on direct expression in eukaryotic cell because of difference in transcription and translational mechanism in two groups.

(2) Since eukaryotic genomes are much larger than prokaryotes a genomic clone from a eukaryote needs to be constructed with

pieces of DNA which are 20-40 kb long. A vector like pBR322 inserts greater than 10-15 kb fails to give satisfactory transformation, also enzyme being glycoprotein will not be glycosylated and secreted in E.coli, which is generally the choice for gene cloning and expression.

The use of lignocellulosics for production of ethanol and other chemicals, feed stocks is one of the most difficult task encountered in history of biotechnology, while several of these studies contributed to a fund of basic information on the genomic organisation of xylanase synthesis by the microorganisms, there is little so far by way of positive result which could be directly applied to an industrial process for obtaining either a xylanase with more desirable attributes or for more-rapid production of enzyme. The detailed studies, like physico-chemical properties, catalytic properties etc., can lead to enzyme engineering (as in case of subtilisin, scientists have used site directed mutagenesis to alter enzyme pH, profile, thermal stability, substrate specificity and even reaction type). New or improved enzymes are playing an increasingly important role in transformation of biomass.

Fungi have so far been the choice organisms as they are able to utilize the biomass in native lignocellulosic form (Schurz, 1977). Among prokaryotes, actinomyctes have been shown to rapidly colonize and extensively penetrate wood tissue possessing cellulase, hemicellulase and lignolytic enzymes (Hagerdahl et al., 1978; Crawford, 1981, Vanzyl, 1985).

Among actinomycetes, Streptomyces are especially attractive candidate because of the genetic knowledge and cloning technology available with the organisms.

Before substantial utilization of Streptomyces for hemicellulose conversion can be successful, more has to be known about the way they decompose, the nature of enzyme involved. Enzyme production cost must be reduced through the use of more efficient production methods and better understanding of xylanase secretion and its regulation. Lastly the search of organism in terms of quality and quantity is an important requirement.

MATERIALS AND METHODS

1. ORGANISM

Two strains of Streptomyces were selected for the study incorporated in this thesis. These strains are listed in Table.

S.No.	Strain	Source
1.	Streptomyces flavogriseus 45CD	Dr. David Klueptel
2.	Streptomyces wedmorensis	ATCC 21230

2. MEDIA AND BUFFERS

(a) Y.E.M.E. medium

Yeast extract	3 gm
Bactopeptone	5 gm
Malt extract	3 gm
Glucose	10 gm
Distilled water	1 litre

Glucose was autoclaved separately at 10 lb/m^2 for 10 min and added to a final concentration of 1%.

(b) Zone clearing medium (Sylvestre-Daigneault and Klueptel, 1979)

$(NH_4)_2SO_4$	1 gm
KH_2PO_4	1.5 gm
K_2HPO_4	5 gm
$MgSO_4 7H_2O$	0.5 gm
KCl	0.5 gm
Trace metal solution	1 ml (Mandel and Sternberg, 1976)

Yeast extract.....	0.5 gm
Agar.....	12 gm
Distilled water	1 litre

Before autoclaving, pH of medium was adjusted to 7.2.

(c) Enzyme release medium (Ishaque and Kluepfel, 1981)

KH_2PO_4	1.5 gm
K_2HPO_4	2 gm
$(NH_4)_2SO_4$	1.4 gm
Yeast extract	2 gm
Protease peptone	1 gm
Tween-80	2 ml
Trace metal solution.....	1 ml (Mandel and Reese, 1957)
Distilled water.....	1 litre

pH was adjusted 7 with NaOH before sterilization. To this medium inducer substrate was added to final concentration of 1% to serve as principal carbon source. After sterilization 30 mg of $MgSO_4 7H_2O$ and 30 mg $CaCl_2$ were added aseptically.

(d) Sodium acetate buffer

0.2 M sodium acetate buffer was prepared in double distilled water. pH of the solution was adjusted 5 with acetic acid.

(e) Tris glycine buffer (10 mM, pH 8.3)

Tris	1.21 gm
Glycine	0.75 gm
Distilled water	1 litre

(f) Sterilization of buffers and media

Buffers and media were autoclaved at 15 lb/in^2 for 10 min.

(g) Solidification of medium

It was achieved by addition of 1.2% agar (oxoid).

3. GROWTH CONDITION AND MAINTENANCE OF CULTURES

Both the streptomyces strains were maintained in sporulated form on Y.E.M.E. agar slants. The cultures were stored at $4^{O}C$ and routinely transferred every 30th day.

When a culture was required for experiment, it was taken from stock culture stored at $4^{O}C$ and streaked on Y.E.M.E. slants incubated at 30^{O} for 5-7 days for good sporulation.

For growth in liquid medium, overnight (O/N) inoculum was prepared by suspending the spores from 5-7 day old slant in 10 ml of LRM without inducer. The incubation was carried out on a rotary shaker with an agitation rate of 240 rpm at $30^{O}C$ for 12 hrs. This was used for inoculating 500 ml Erlenmeyer flasks, each containing 100 ml of medium with 2 ml of spore suspension and further incubating at $30^{O}C$ on a rotary shaker.

4. ENZYME PREPARATION

(a) Extracellular enzyme fraction

Samples of culture were taken at regular intervals throughout the growth phase. The xylanase activity was measured by following

the release of reducing sugar from xylan. The culture supernatant was collected by pelleting the cells at 6000 x g for 5 min, this supernatant was washed with 200 mM sodium acetate buffer pH 5 in ultrafiltration unit using a membrane filter having a cut off value of 10 k dalton before use to remove low molecular weight compounds, this was lyophilized and redissolved in appropriate buffer. This was treated as extracellular enzyme fraction.

(b) Intracellular enzyme fraction

Pellets were washed twice with 0.2 M sodium acetate buffer pH 5 and resuspended in same buffer. Cells were sonicated for 10 min in ice in presence of 0.1 mM PMSF (phenyl methyl sulfonyl fluoride, Boehringer, West Germany) with 2 min interval (Cell disrupter model W-220F, Heat systems ultrasonic Incorporation, USA) operated at 20 kC/sec.

The sonicated extract was centrifuged at 15,000xg for 30 min at $4^{o}C$, the supernatant and pellets were treated as intracellular and cell bound fractions.

5. PROTEIN ESTIMATIONS

Proteins were estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard. Following reagents were used:

(i) Reagent I: 8% Na_2CO_3 in D.D.W.

(ii) Reagent II: 300 mg $CuSO_4.5H_2O$
600 mg sodium potassium tartarate dissolved in 500 ml D.D.W.

(a) Protein estimation

For protein estimation, 0.5 ml of aliquot was mixed with 5 ml of test reagent (Reagent I and II in 1:1 ratio as described above) and incubated at 37°C for 10 minutes, 0.5 ml of 1N Folin's reagent was added in the mixture vortexed and kept in dark for 30 min, optimal density of blue coloured complex was measured at 625 nm. Protein was estimated from the standard linear curve obtained from bovine serum albumin.

(b) T.C.A. precipitation

Proteins were precipitated with 10% trichloroacetic acid according to Lowry et al. (1951). 0.2 ml of aliquot was mixed thoroughly with 2 ml of 10% TCA kept for 3-4 hrs in cold, then centrifuged at 2000 rpm for 15 min washed with 0.2M sodium acetate buffer pH 5, dried and finally dissolved in 0.2 ml of 0.1N NaOH, protein in the above fraction was estimated by Lowry's method as described above.

6. XYLANASE ASSAY

The assay mixture contained 0.5 ml of appropriately diluted enzyme preparation and 0.5 ml of 1% xylan suspension in 0.2 M sodium acetate buffer pH 5 incubated at 40°C for 10 min blank consisted of 0.5 ml of 1% xylan suspension and 0.5 ml of culture filtrate at 0 min. The reaction was terminated by boiling for 15 min and 1.5 ml of DNS (Dinitro Salicylic) reagent was added. The amount of reducing sugar released was determined according to Miller

et al. (1960) with D-xylose as standard, absorbance of the supernatant was measured at 575 nm against the blank.

A conventional unit to define the activity of enzyme is the amount of enzyme that catalyzes the release of umole of reducing sugar as xylose from substrate per millilitre per minute at the optimum temperature.

7. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

(a) Non-denaturing gel

Slab gel electrophoresis was performed at room temperature (Studier, 1973) stacking gel containing 2.5% and separation gel containing 7.5% acrylamide were prepared from stock solution containing 30% acrylamide and 0.8% N,N' methylene-bis-acrylamide. The stacking gel solution was 0.061M Tris (pH 6.8) and separation gel solution was 0.377M Tris (pH 8.8). The running buffer consisted of 0.025M Tris and 0.192M glycine, pH 8.3.

Enzyme sample prepared as described in Section 4, was redissolved in electrophoresis buffer, approximately 100 ug protein measured after TCA precipitation was loaded in two lanes in non-denaturing gel. Gel was run at 200V for 20 min after stacking of the samples, the voltage was decreased to 150 volts. Gels were electrophoresed for a period of 5 hrs and Bromophenol blue was used as a tracker dye. Following electrophoresis, the gel was removed, one lane was stained with Coomassie blue and other lane was used for detection of endoxylanase activity as described in Section 7C.

(b) Denaturing gel

All conditions and reagents were same as in non-denaturing gel except that 2% and 0.1% sodium dodecyl sulphate (SDS) was present in sample and running buffer respectively. The protein sample was suspended in sample buffer and kept at 37°C for 90 sec before loading on the gel. The lane used for detection of endo-xylanase activity was washed four times for 15 min in 0.2 M sodium acetate buffer pH 5. The first two washes contained 25% isopropanol.

(c) Zymogram staining for detection of endoxylanase activity

Polyacrylamide gel was placed in dish. An agar substrate gel poured separately, was placed on top of the polyacrylamide gel and dish was placed in water bath at 50°C for 1 hr. The agar substrate gel consisted of 0.1% xylan 2% (w/v) agar in 0.2M sodium acetate buffer pH 5. Following incubation, the substrate gel was removed, washed with 0.2M sodium acetate buffer, pH 5 and immersed in 0.1% (w/v) Congo red for 30 min, followed by washing in 1M NaCl. The stained substrate gel was stored in 10% acetic acid or 1M NaCl (Teather and Wood, 1982).

8. CHARACTERIZATION OF XYLANASE

(a) Optimal temperature for enzymatic hydrolysis

Optimal temperature of enzyme was determined by measuring the initial rates at which reducing sugars are released at different temperatures ranging from 20-60°C.

The reaction mixtures were incubated at different temperatures for 30 min and the reducing sugar released was determined as described in Section 6.

(b) Optimal pH determination

For determination of the pH range at which xylanase is active, five different buffers were made covering the range between 4 to 8. Xylanase activity was assayed at 30 and 60 min interval.

(c) Thermal inactivation of enzyme

Samples of enzyme were pre-incubated at 40^{O}, 50^{O} and $60^{O}C$ respectively. At the given time interval, i.e. 1hr, 2hr, 3hr, 5hr, 12hr and 24 hrs, aliquots were assayed for enzyme activity at $40^{O}C$ for 30 min.

(d) Inducers for xylanase production

To determine whether the strain would produce xylanolytic activity if grown on substrate other than xylan, cells were grown in ERM supplemented with one of the variety of sugars i.e. maltose, xylose, mannose sucrose, fructose, glucose, cellobiose, lactose, arabinose, cellulose, avicel, carboxymethyl cellulose, galactose and glycerol to the final concentration 1%. Supernatants were assayed for xylan degrading activity after time interval of 12hr, 60hr, 80hr and 108hr respectively.

(e) Kinetic parameters

Determination of Km and `Vmax of crude culture filtrate was carried out by incubating fixed amount of enzyme with increasing concentration of substrate ranging from 0.1 mg/ml - 3 mg/ml at $40^{O}C$ for 15 min followed by assay for xylanase activity.

(f) Effect of detergents

SDS and Triton X-100 were added to standard xylanase assay

to final concentration of .1% and 1% respectively to observe their effect on xylan degradation. This reaction mixture was incubated at 40°C for 30 min and xylanase activity was measured.

(g) Effect of inhibitors

Various divalent cations and metal chelators were added to the standard xylanase assay to observe their effect on xylanolytic activity. The cations used were Ag^{+2}, K^{+2}, Ca^{+2}, Mg^{+2} added to the final concentration of 100 mM and 10 mM respectively, whereas EDTA was added to the final concentration 100 mM and 200 mM respectively.

9. SCREENING OF XYLANOLYTIC STRAINS

Different Streptomyces strains were screened on ZCM medium containing xylan as carbon source. Xylanolytic activity was measured by determining the magnitude of clear zone formed in opaque xylan medium.

10. EFFECT OF INCREASING ENDOPRODUCT CONCENTRATION

Xylose added at various concentration from 0-5% allows the detection of concentration at which the catabolic repression occurs. ZCM plates streaked with the Streptomyces strain were incubated at 30°C for 3-15 days, xylanolytic activity was measured by determining the magnitude of clear zone formed in medium. The zone of hydrolysis can be seen with naked eye but it was enhanced by staining with Congo red (0.1%) for 30 min and the excess stain

was washed with 1M NaCl. A yellow zone of hydrolysis is seen around the colony caused by degradation of -1,4-glycosidic bonds of xylan. This assay is a semiquantitative way of determining the amount of enzyme produced (Sylvestre-Daigneault and Kluepfel, 1979).

RESULTS

1. SCREENING OF STREPTOMYCETE STRAIN PRODUCING HIGH LEVELS OF ENDOXYLANASE

In view of our objective to isolate highly xylanolytic Streptomyces sp., several strains available in laboratory were screened they were as follows:

(1) Streptomyces wedmorensis ATCC 21230

(2) Streptomyces flavogriseus 45CD

(3) Streptomyces graminifaciens ATCC 12705

(4) Streptomyces lividans

(5) Streptomyces sp. ATCC 21175

(a) Primary screening

The above strains were screened for xylanolytic activity by growing them on zone clearing medium containing 0.5% xylan. They were incubated at 30°C for 5-7 days and the xylanolytic activity was detected as clearing zone around the streptomyces colonies on opaque xylan medium, the sensitivity of this method is increased in presence of 0.2 M sodium acetate buffer pH 5 which was added to plates after third day and zone of clearing was observed on fourth day (Sylvestre-Daigneault and Kluepfel, 1979). To further enhance the clearing, the plates were flooded with aqueous solution of Congo red (1 mg/ml) for 30 min, then washed with 1M NaCl for 15 min, the zone of hydrolysis could be clearly visualized (Teather and Wood, 1982). The strains were selected on the basis of above screening because xylanolytic enzymes are released from culture

then only zone of clearing is produced by degradation of β1,4-glycosidic bonds of xylan. The screening thus allowed selection of xylanolytic strain with extracellular enzyme production.

(b) Secondary screening

The second basis was the ability of the strains to grow on solid media with xylan as sole carbon source, on the basis of above two screenings S.flavogriseus and S.wedmorensis were selected for further studies.

Characterization of streptomycete IAF 45CD S.flavogriseus produces grey aerial mycelium mass on all the media studied and sporulated abundantly on most of them. The spore chain morphology is typically rectiflexibilis and consists of upto 10 spores. The conidia have smooth surface as determined by electron microscopy. The strain grows optimally at 30°C with temperature range of 20-37°C. The reverse side pigment of mycelium is distinctively yellow on Y.E.M.E. medium but no soluble pigments such as melanin are produced.

Xylanolytic activity in submerged cultures - Strains were grown in ERM + 1% xylan (Sigma, U.S.A.) and assayed for xylanase production. The strains were incubated at 30°C for 3 days and samples were taken every 12 hrs.

S.flavogriseus - Maximum activity in terms of enzyme unit i.e. 45 I.U. was produced in filtrates of strain after 2 days of incubation. In 72 hr sample, the enzyme activity was found to decrease slightly

though in 86 and 98 hr sample it was relatively constant. The time course of xylanase production in S.flavogriseus is shown in Fig. 3 . Xylanase activity in medium increased proportionally to cell growth, reached its maximum 48 hrs after inoculation and thereafter remained almost constant. Specific activity was found to be 27.5 I.U. per mg of protein.

The extracellular filtrates contained only trace amounts of β -xylosidase. The intracellular xylanase levels were very low as compared to extracellular levels. The cellulose system is inducible by xylan as primarily shown by Kluepfel and Ishaque (1982).

S.wedmorensis - The growth of the strain was followed in enzyme release medium, containing xylan (1%) as substrate at 30°C and 37°C. The strain grew well at both the temperatures and produced maximal enzyme levels after 72 hrs of incubation at 30°C and 56 hrs at 37°C yielding 2.25 to 2.5 mg/ml of reducing sugar in supernatant consistently. In terms of enzyme unit 39 I.U. were obtained at 37°C after 56 hrs and 35 I.U. at 30°C after 72 hrs. However, the growth was better at 30°C. At 30 and 37°C no intracellular xylanase activity was found. During growth pH was not controlled it remained 7 throughout.

(c) Inducers of xylanase

It was of interest to determine whether the strains would produce xylanolytic activity grown on carbon source other than

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(c) Inducers of xylanase

It was of interest to determine whether the strains would produce xylanolytic activity grown on carbon source other than

xylan. Accordingly, the strains were grown in ERM supplemented with one of the variety of sugars. Supernatants were assayed for xylanase activity at 12, 24, 36 and 72 hrs respectively.

In S.flavogriseus, of all inducers tested xylan, lactose, mannose and glactose were the most effective ones. The highest xylanase activity was obtained on xylan and lactose, yeast extract was also effective for xylanase production. An initial pH 6.0-7.0 and temperature 30°C was maintained in all conditions. There was no xylanase production when strain was grown in medium containing glucose as sole carbon source. After 48 hrs, xylan and lactose were found to be the most effective inducers. Mannose and galactose also induced activity but to lower extent, with cellulose the activity reached maximum after 60 hrs, while glycerol, sucrose, fructose, arabinose, xylose, cellobiose, maltose and glucose were found to be very poor inducers as there was no increase in activity with time.

The protein content was found to increase in first 12 hrs with xylan and lactose as inducer and there was considerable decrease after that, though later on it was almost found constant, as far as with other inducers, protein concentration was seen ascending for 20 hrs and then over next 12 hrs there was a heavy decline which was later almost constant.

S.wedmorensis - Several carbon sources were tested to find the best inducer, xylan (1%) was recognized as the best inducer.

Other effective inducers included cellulose, lactose, D-glactose and D-mannose (Fig.\\ ). With D-arabinose, xylose, maltose, glucose sucrose and fructose the xylanase activity was at basal level and it did not rise with time.

The protein content followed the same pattern as that of S.flavogriseus with xylan, cellulose, mannose, lactose and galactose as inducers, there was an initial increase for first 12 hrs, which decreased slightly over next 8 hrs and this remained constant upto 60 hrs, as for rest of the inducers protein content was found to be almost constant 12 hr onwards.

(d) Optimal temperature for enzymatic hydrolysis

The optimal temperature in 30 min assay with soluble larch xylan as substrate at pH 5 were $50^{o}C$ and $40^{o}C$ for S.flavogriseus and S.wedmorensis respectively.

(e) Optimal pH for enzymatic hydrolysis

For determination of pH range at which xylanase is active, four different buffers were used covering the range between 4-8, xylanase activity was assayed at 30 and 60 min intervals.

S.flavogriseus - At both time intervals, maximal activity was obtained at pH 6 though enzyme retained 95% of its activity at pH 7. There was a steep decline in activity in alkaline side (pH 8).

S.wedmorensis - At both time intervals, maximal activity was obtained at pH 7 but the enzyme retained over 98% activity at

pH 8. The enzyme reaction was carried out in buffer pH 7 and similar in both the cases.

(f) Thermal inactivation of enzyme

S.flavogriseus - The stability of enzyme against thermal inactivation is shown in Fig.9 . Exposure at 30°C upto 48 hrs hardly affected the enzyme, whereas at 40°C, the half life was 24 hrs and at 50°C the half life was further reduced to 2.5 hrs.

S.wedmorensis - The stability of enzyme against thermal inactivation is shown in Fig.15 . Samples of enzymes were preincubated at the temperatures indicated in figure. At the given time interval, aliquots were assayed for enzyme activity at 40°C for 30 min. Exposure at 40°C hardly affected the enzyme activity even after 48 hrs. The enzyme had significant stability at 50°C, 75-79% activity remained after 48 hrs of pre-incubation at 50°C. Even at 60°C the half life was about 5 hrs.

(g) Substrate stabilization

S.flavogriseus - Considerable substrate stabilization was observed at 50°C, in presence of substrate the enzyme was dramatically more stable, half life was extended from 2.5 hrs to 7 hrs. Activity levels in the presence of substrate were calculated on the basis of reducing sugar released between adjacent time points.

Similarly at 40°C the half life was increased from 24 hrs to 40 hrs. For S.wedmorensis the half life of xylanase was extended from 5 hrs to 9 hrs at 60°C.

(h) Inhibition by cations, chelators and detergent

Various divalent cations, metal chelators and detergents were added to standard xylanase assay to observe their effect on the xylanolytic activity of strain supernatant. It was observed that S.flavogriseus the enzyme activity was inhibited significantly by 200 um of Hg^{2+}, however, there was no inhibition by 20 um whereas with 200 um there was total loss of activity, other cations at the concentration of 200 um showed no significant inhibitory effect. There was overall lack of inhibition by these metals except at very high concentrations.

With EDTA no inhibition was observed at 1 mM concentration whereas 50 mM and 100 mM resulted in only 5% and 12% inhibition respectively.

With detergents i.e. SDS and Triton X-100 at a concentration of 1% there was considerable inhibition.

S.wedmorensis xylanase was comparatively found to be more sturdy as with 20 um of Hg^{2+} there was no inhibition but 200 um there was 85% inhibition which is 10% less than S.flavogriseus and similarly for Ca^{+2}, Mg^{+2} and Ag^{+2} at concentration of 200 um there was no significant inhibitory effect.

(i) Production inhibition

S.flavogriseus - Inhibition of xylanase by addition of xylose was studied by adding varying concentrations of xylose in ZCM + xylan

(0.5%) plates. The strains were grown on them, it was possible to assess rapidly not only the degree of catabolic repression of xylanase production by xylose but also in semiquantitative way the amount of enzymes produced, xylose was added at varying concentration (0-2.5%). This variation allowed the detections of xylose concentration at which catabolite repression occurred. The petriplates were incubated at 30°C for 3-12 days and examined regularly for zone clearing around the colony.

ZCM + Xylose (0.1%) -- no clearing

ZCM + Xylose (0.5%) + Xylan (0.5%) -- large zone of clearing

ZCM + Xylose (1%) + Xylan (0.5%) -- zone of clearing half of the above

ZCM + Xylose (2.5%) + Xylan (0.5%) -- no zone of clearing

ZCM + Glucose -- no clearing

ZCM + Glucose (1%) + Xylan (0.5%) -- narrow zone of clearing

ZCM + Glucose (2.5%) + Xylan (0.5%) -- very narrwo zone of clearing

S.wedmorensis xylanase activity was characterized on polyacrylamide gel (Fig. 2) distinct endoxylanase activity band was detected in crude enzyme preparation of S.wedmorensis in non-denaturing polyacrylamide gel which indicates that xylan degrading activity in S.wedmorensis is true xylanase activity.

The activity was lost on boiling hence enzyme could not be electrophoresed in SDS denaturing gel and zymogram staining done to locate enzyme activity band.

Table-1

Effect of cations chelating agents and detergents S.flavogriseus.

Inhibitors	Concentrations	% Inhibition
Hg^{2+}	20 um	0
	200 um	94
Cu^{2+}	200 um	0
	1 mM	25
Ag^{2+}	200 um	20
Mg^{+2}	10 mM	7
EDTA	50 mM	5
	100 mM	12
SDS	0.1%	33
Triton X-100	0.1%	42.5

Table-II

Effect of cations, chelating agents and detergents S.wedmorensis.

Inhibitors	Concentrations	% Inhibition
Hg^{2+}	20 um	0
	200 um	85
Cu^{2+}	200 um	0
	1 mM	18
Ag^{2+}	200 um	25
Mg^{+2}	10 mM	10
EDTA	50 mM	0
	100 mM	5
SDS	0.1%	27.4
Triton X-100	0.1%	13.2

Fig. I Detection of endoxylanase activity in Streptomyces wedmorensis on zone clearing medium containing xylan [0.5%] after staining with Congo re

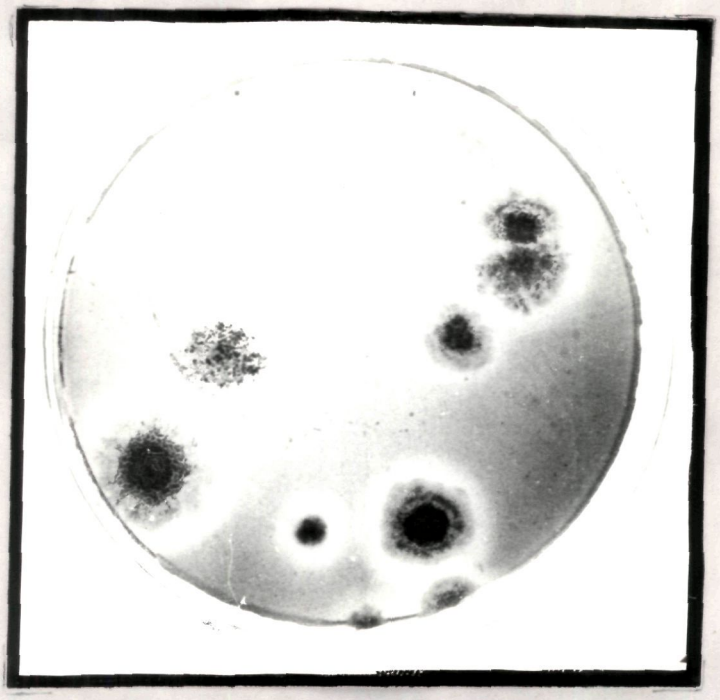


Fig. II SDS polyacrylamide gel electrophoresis of crude endo-xylanase preparation from Streptomyces wedmorensis.

lane (1) Coomassie blue staining
lane (2) Zymogram staining

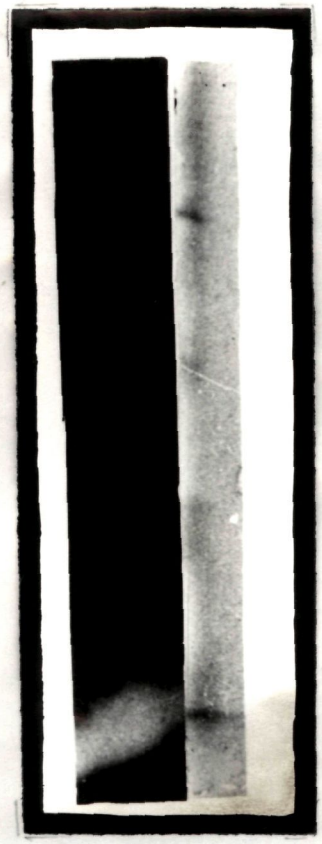


Fig. 3

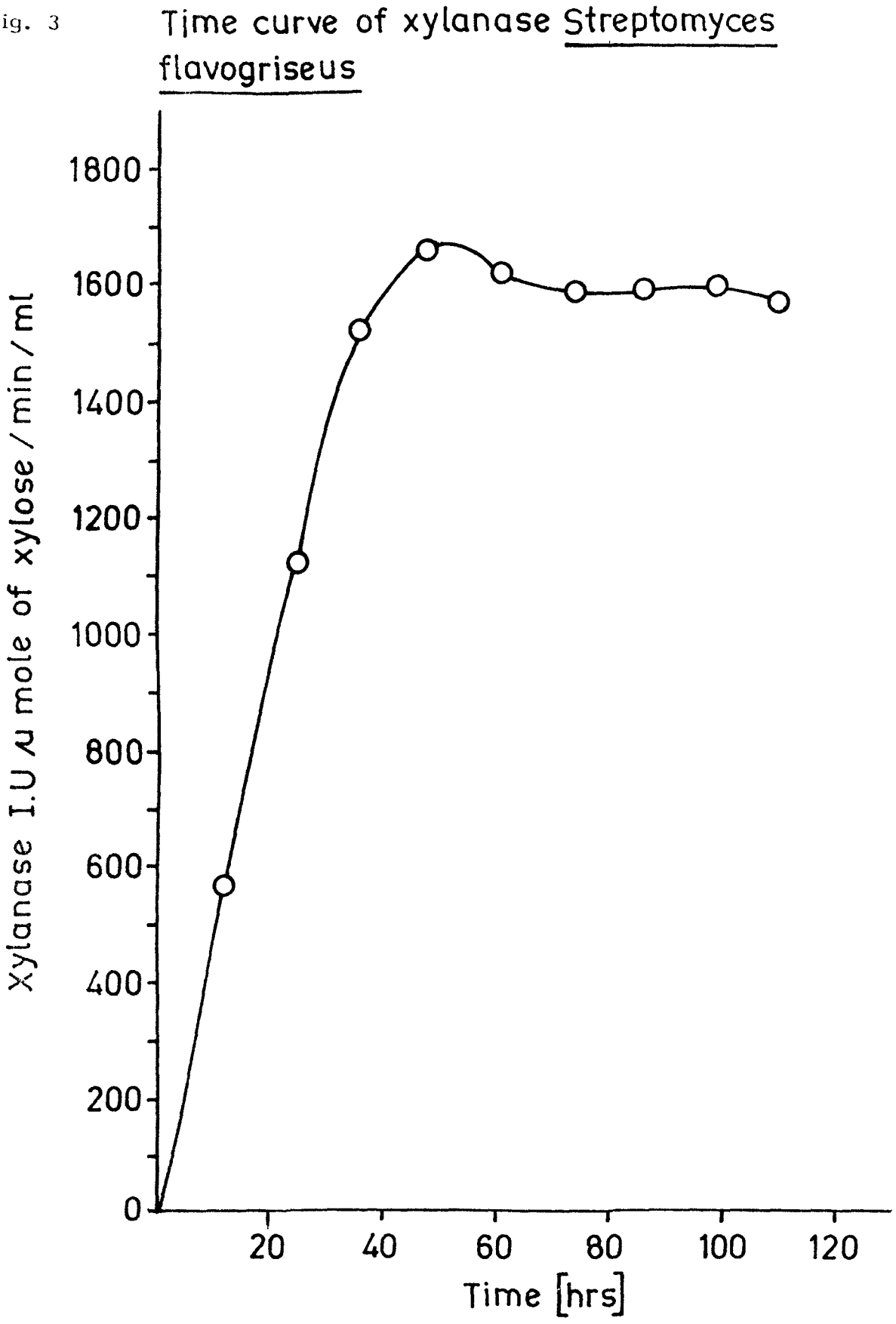

Time curve of xylanase Streptomyces flavogriseus
Xylanase I.U ≙ mole of xylose / min / ml
1800
1600
1400
1200
1000
800
600
400
200
0
20
40
60
80
100
120
Time [hrs]

Fig. 4 Inducers for xylanase of Streptomyces flavogriseus

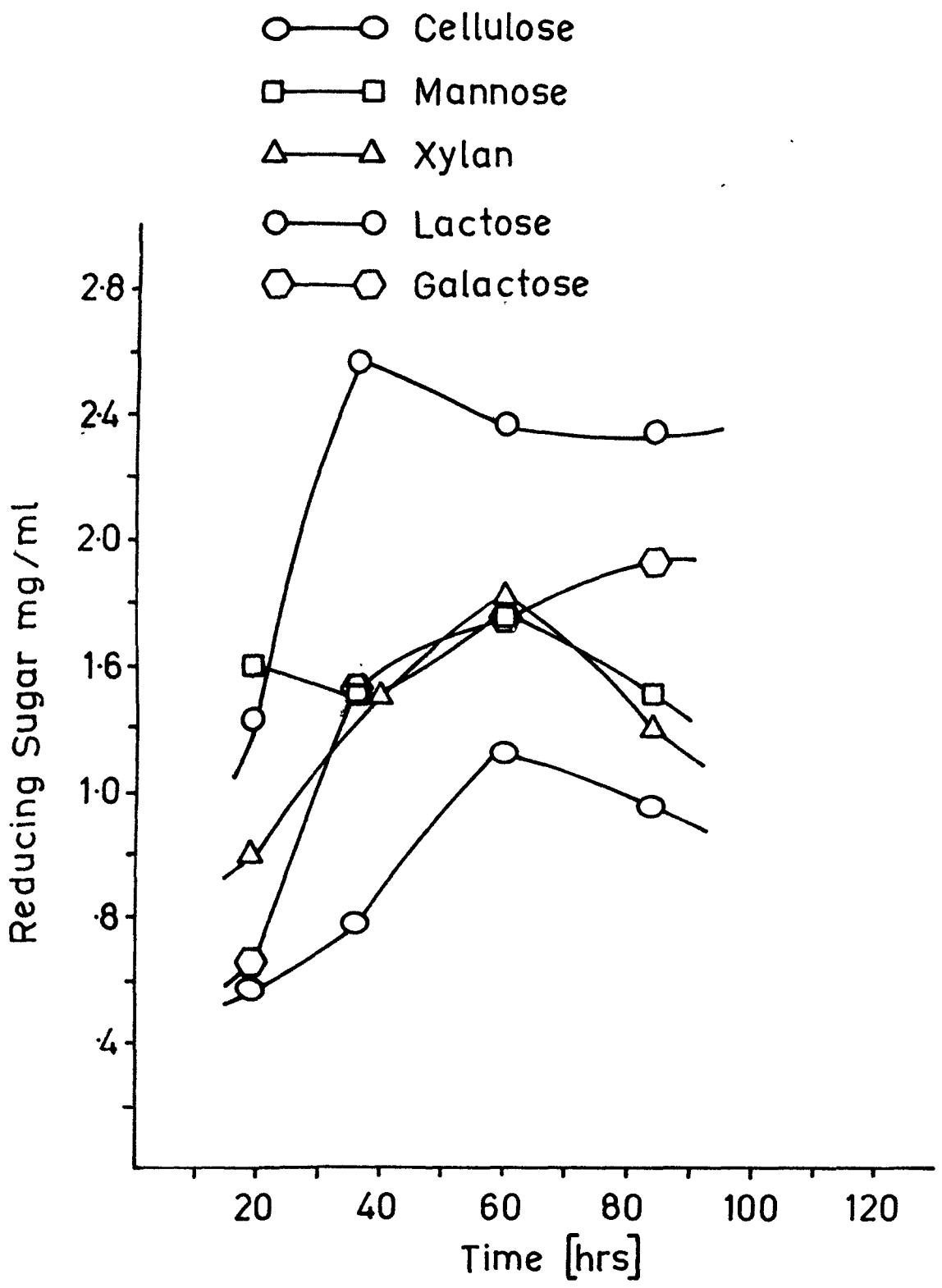


Fig. 5 Inducers for xylanase of Streptomyces flavogriseus

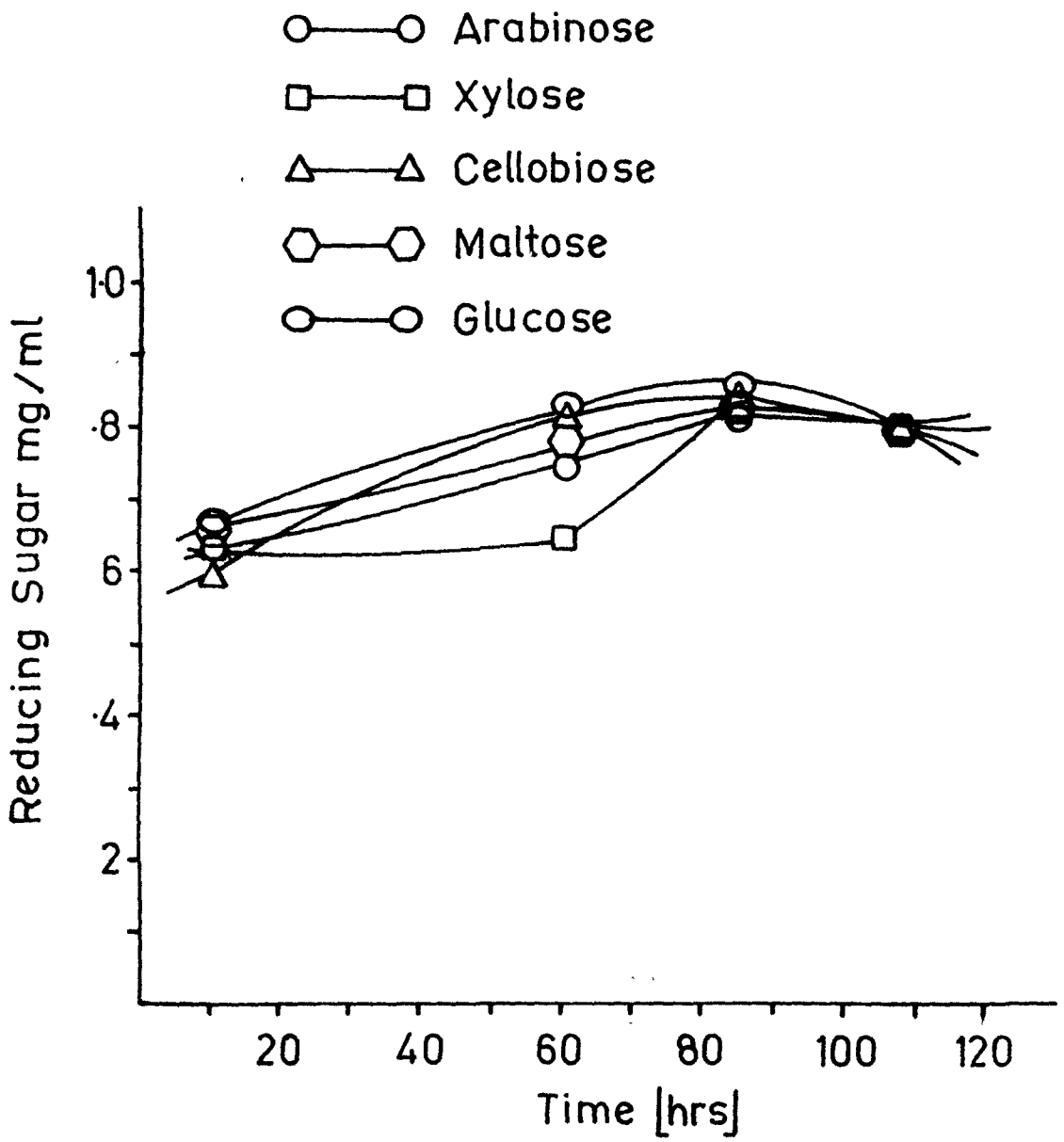


Fig. 6 Inducers for xylanase of Streptomyces flavogriseus

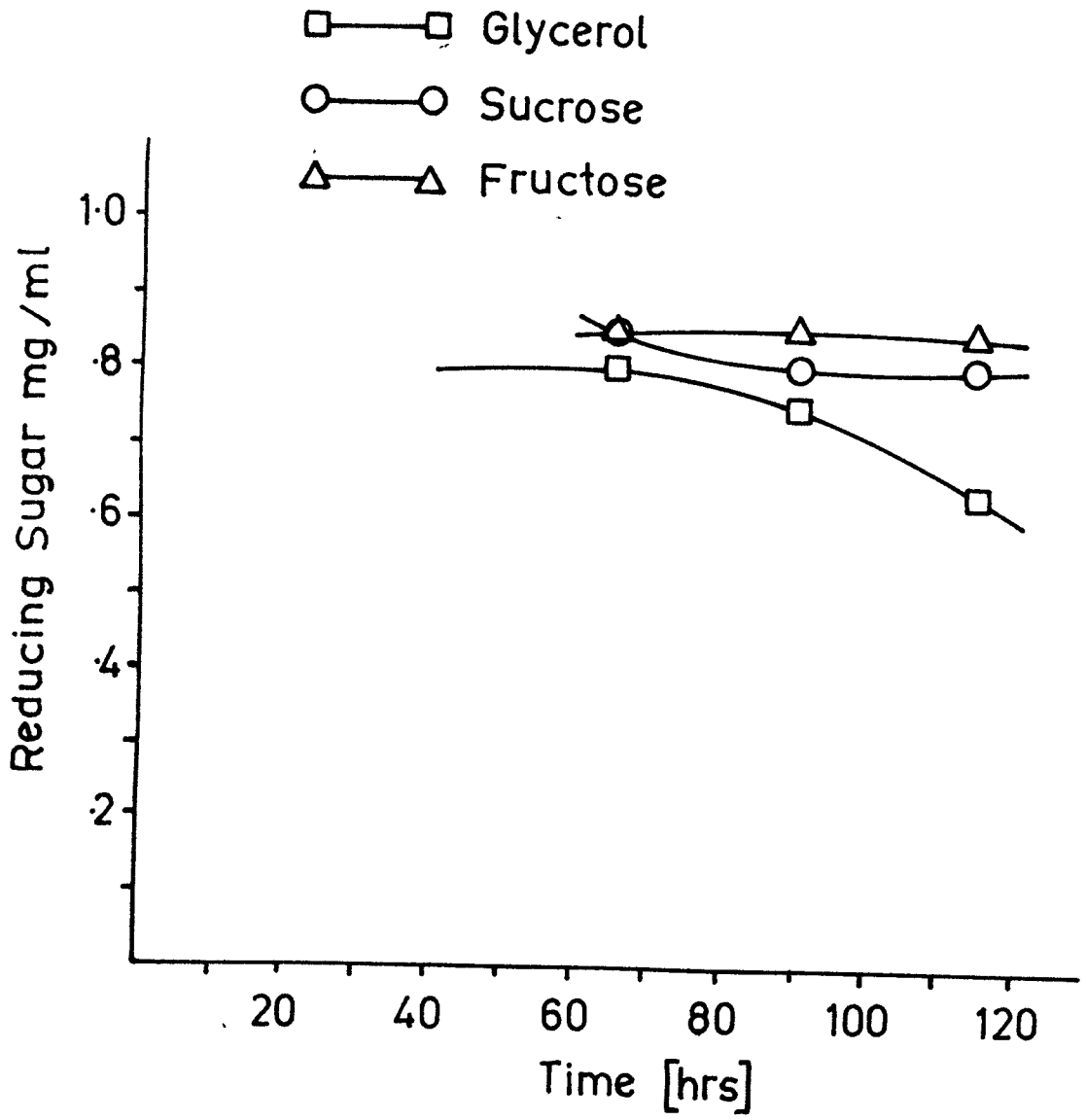


Fig. 7 Optimal temperature for xylanase of Streptomyces flavogriseus

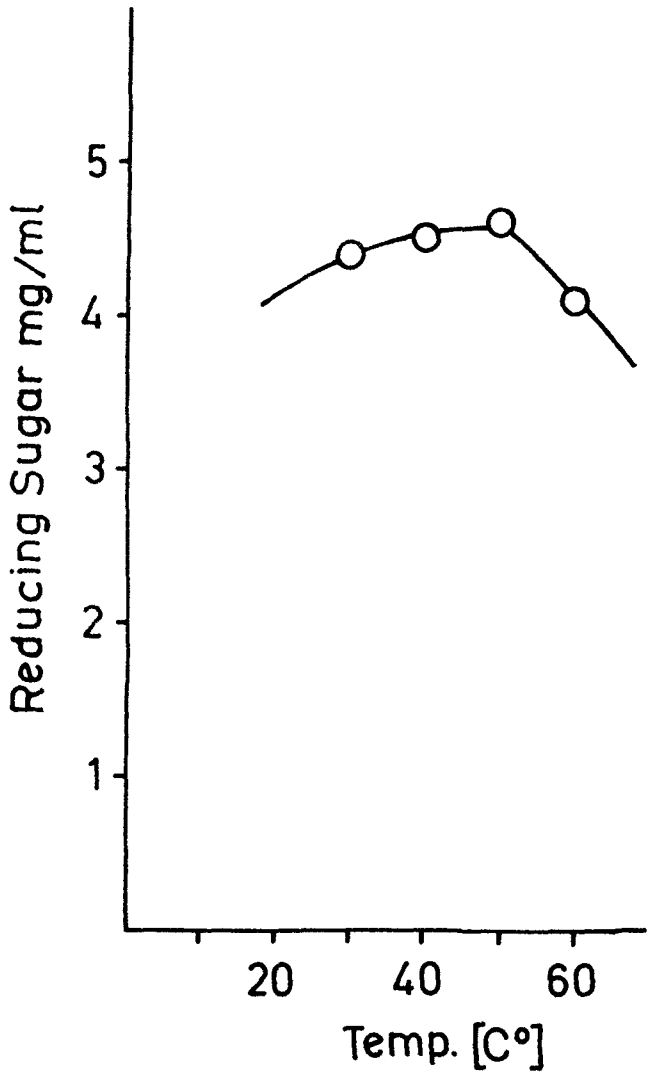


Fig. 8 Optimal pH for xylanase of Streptomyces flavogriseus

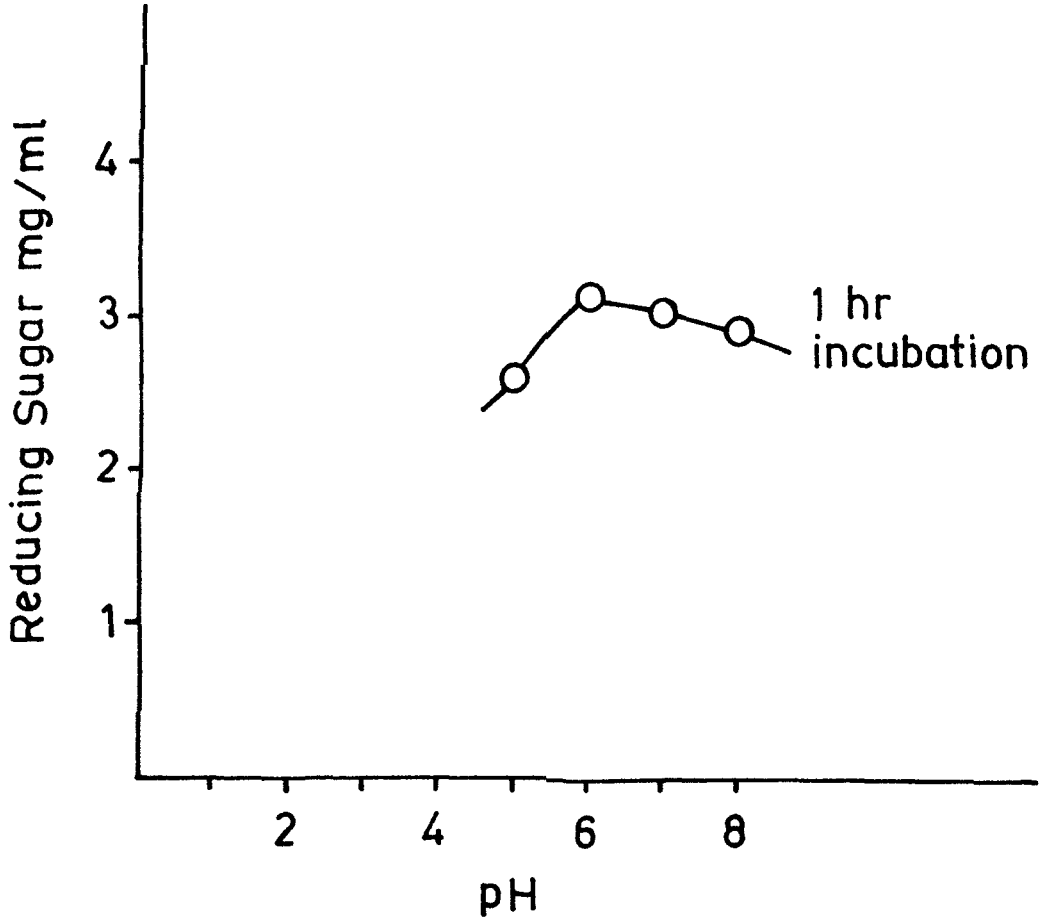
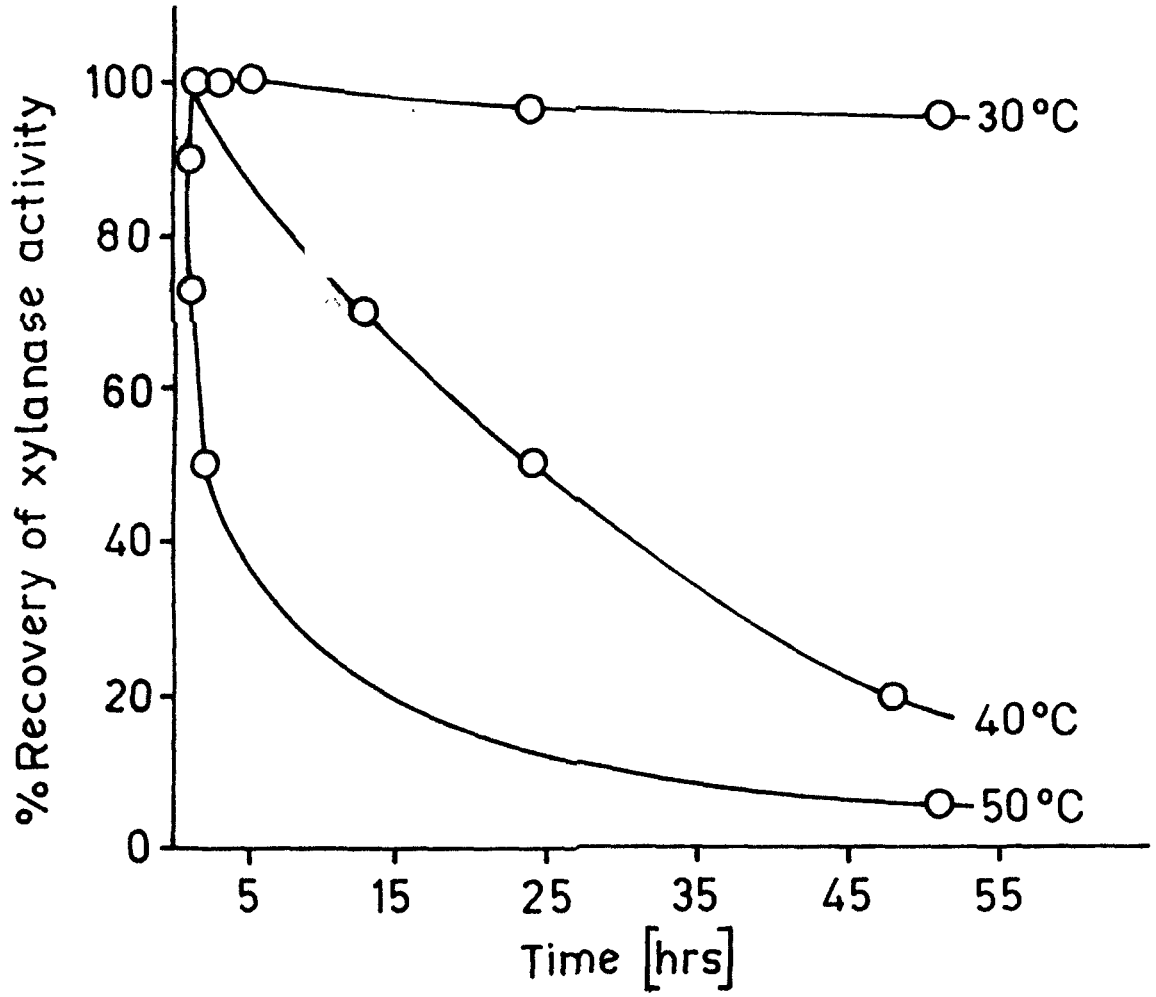


Fig. 9 Thermal inactivation of Streptomyces flavogriseus



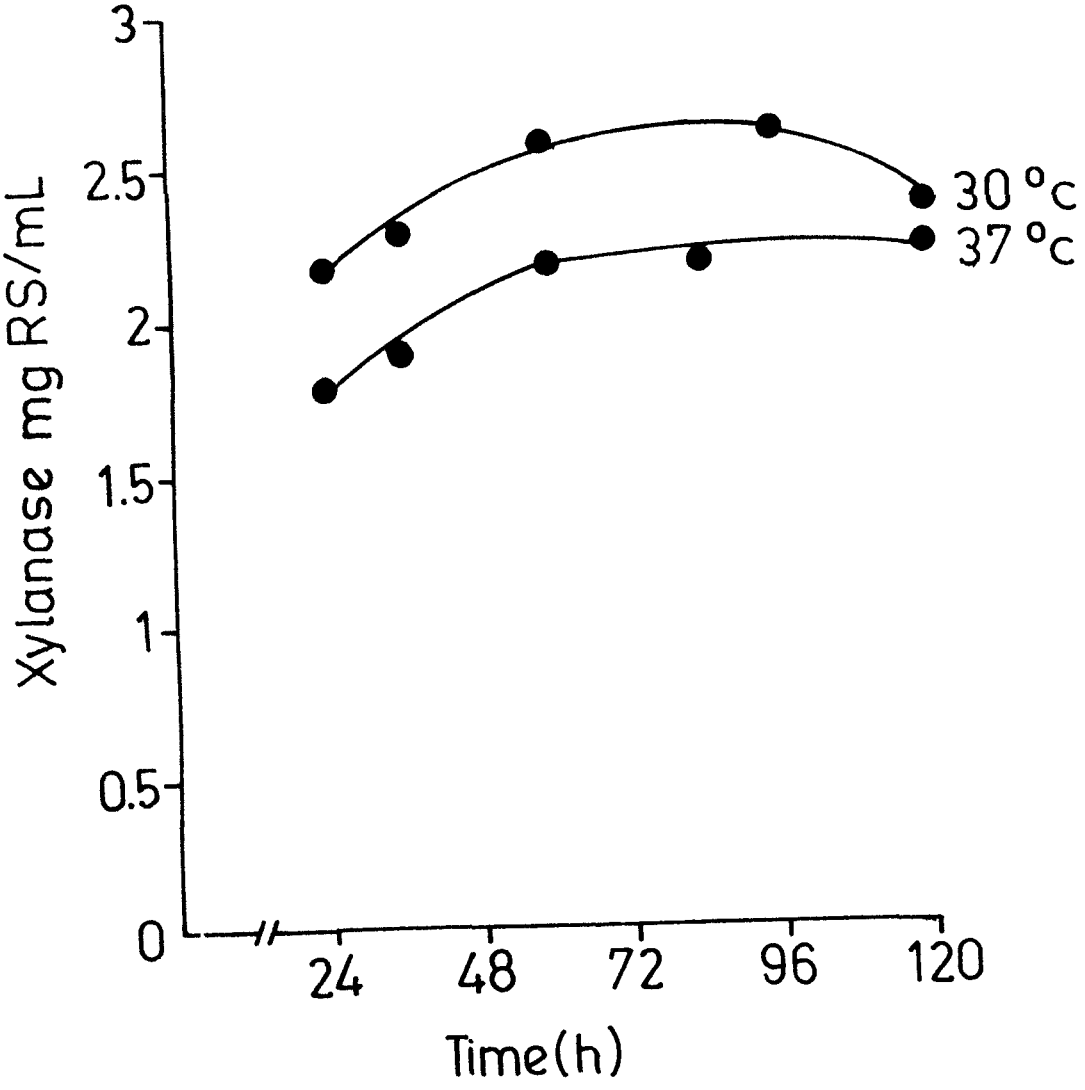


Fig. 10 Production of extracellular endoxylanase on xylan medium at different temperatures in S. wedmorensis.

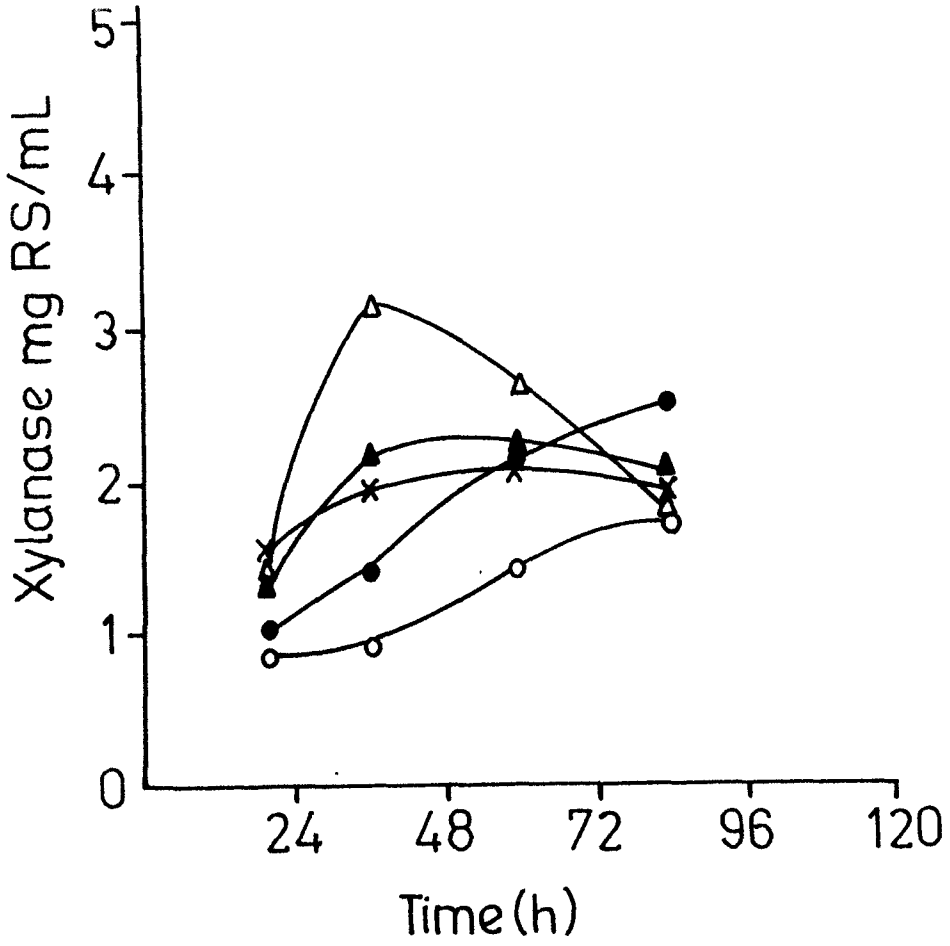


Fig. 11 Induction of xylanase activity during growth on xylan [●], Lactose [■], Mannose [△], Galactose [□] and cellulose [o] in S. wedmorensis.

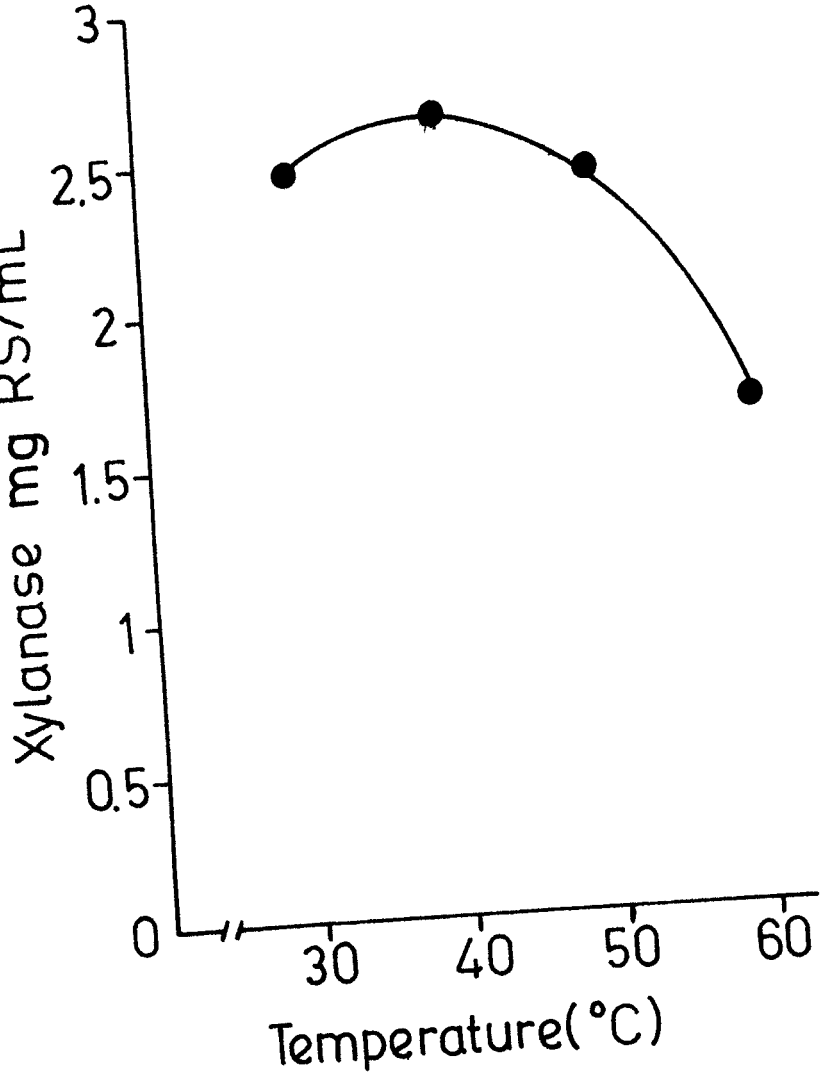


Fig. 12 Influence of temperature on the S.wedmorensis xylanase activity.

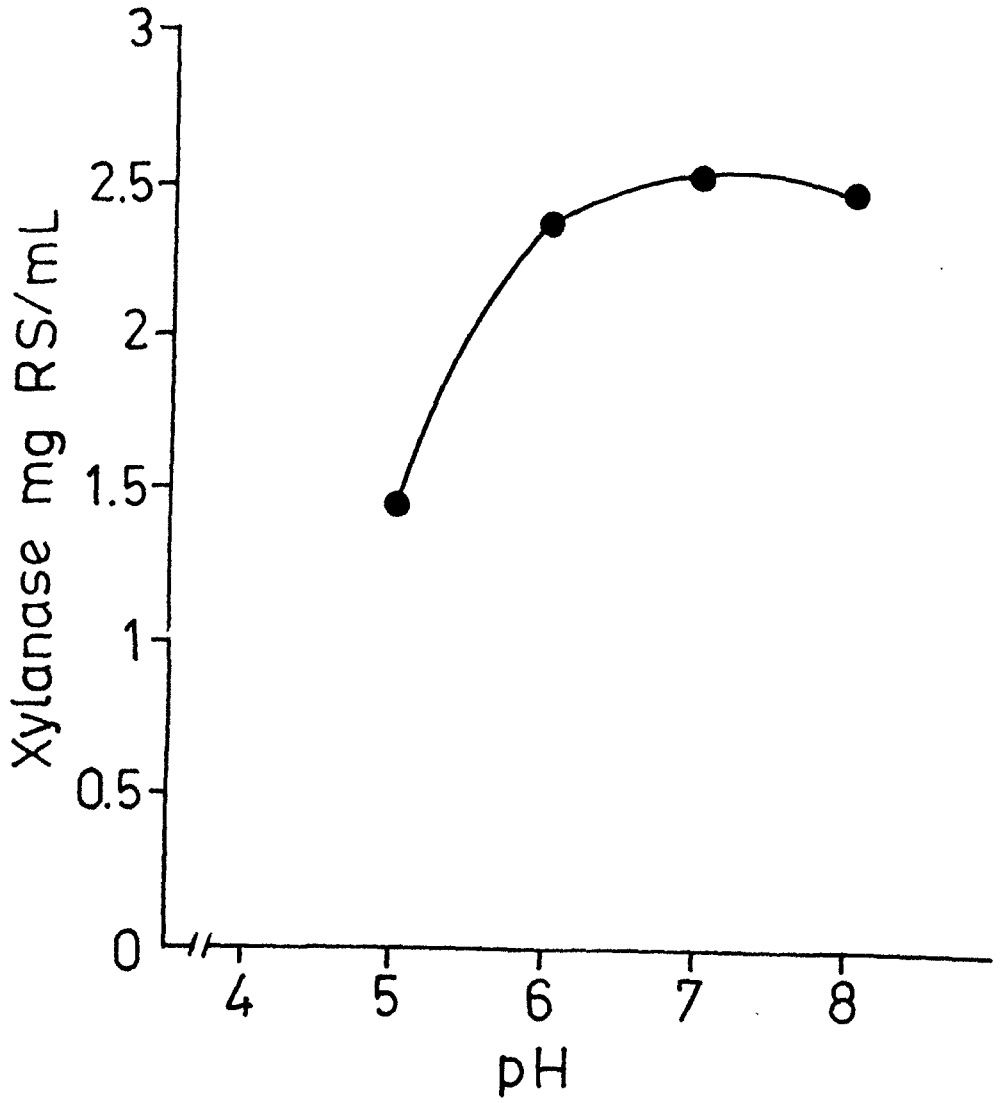
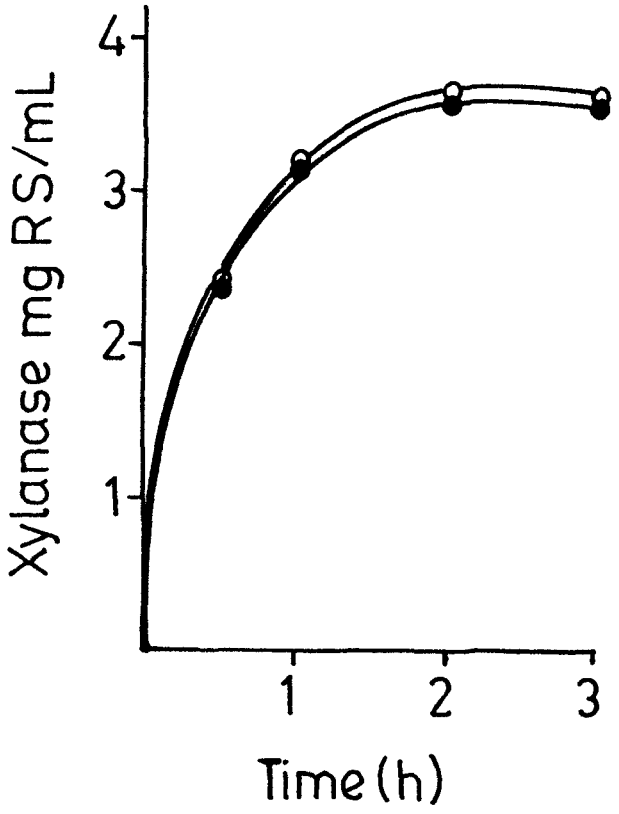


Fig. 13 pH profile of S.wedmorensis xylanase.



} 14 Influence of pH 7 [o] and pH 8 [•] on S.wedmorensis xylanase activity.

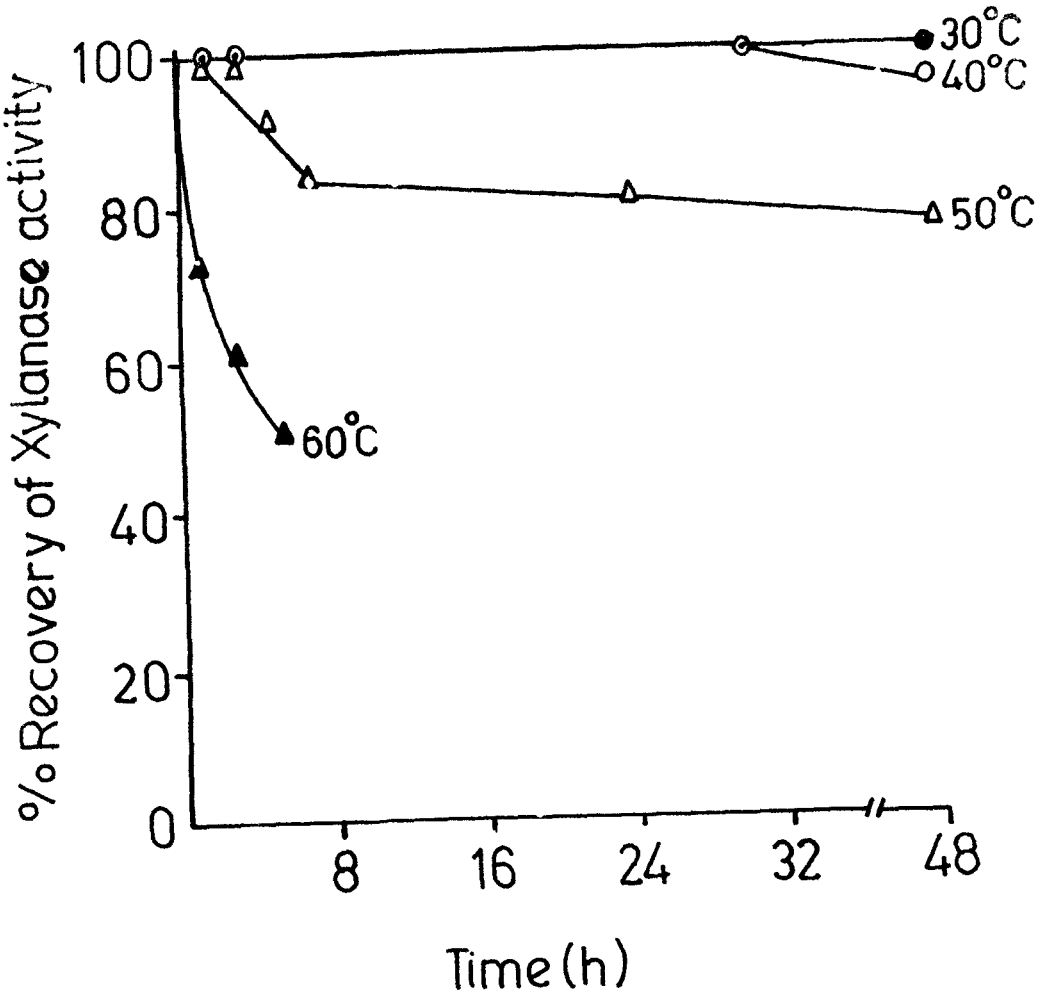


Fig 15 Thermal stability of S. wedmorensis xylanase

DISCUSSION

Xylanase activity has earlier been characterized in detail from several Streptomyces strain (Ishaque and Kluefel, 1982; Marui et al., 1985; Kluepfel et al., 1986), while screening a variety of streptomyces for xylanolytic activity by a previously described method (Daigneault-Sylvestre and Kluepfel, 1979). The strains were selected on the basis of higher magnitude of zone of clearing on agar xylan medium. S.wedmorensis and S.flavogriseus produced clearing around the colonies, this indicated the synthesis of extracellular xylanase and led to further investigation of strains.

When the cultures of mesophilic streptomyces strains were grown on ERM containing xylan (1%) in shake flasks, it produced considerable amounts of extracellular xylanase. The extracellular proteins and xylanases were closely related and proportional, the growth was rapid reaching its maximum in 48 hrs, the optimal growth temperature was found to be 30°C though the strains were able to grow at 37°C. In terms of enzyme unit, S.flavogriseus produced 45 I.U. after 48 hrs and S.wedmorensis produced 35 I.U. after 72 hrs, maximum yields of xylanase upto 200 I.U. by a basidiomycete Sclerotium rolfsii have been reported by Sadana et al. (1980). These values were obtained from 14 day old cultures. Thus the 45 I.U. and 35 I.U. of xylanase activity produced in filtrates of S.flavogriseus and S.wedmorensis after two and three days of incubation compare favourably with Sclerotium rolfsii.

The comparison of xylanase production at different temperatures viz. 30°C and 37°C in S.wedmorensis is presented in Fig.

Maximum xylanase production was obtained at 30°C after 72 hrs and at 37°C after 56 hrs. Xylanase production in S.wedmorensis followed patterns that were clearly different than those reported previously for S.flavogriseus (Ishaque and Kluepfel, 1980) where maximum enzyme synthesis was observed at 30°C. S.wedmorensis had a wider temperature range than that of strict mesophile S.flavogriseus.

The specific activity for xylanases of S.flavogriseus and S.wedmorensis was found to be 27.5 I.U. per mg of protein and 29 I.U. per mg of protein which are comparable with results obtained from S.lividans and S.flavogriseus (Marui, 1986; Kluepfel, 1982).

Xylanase activity was localized in both the strains and found extracellular in nature. Approximately 80% activity was released into the medium, intracellular xylanase levels were very low compared with extracellular activity.

Both strains produced β 1,4 endoxylanase and xylosidase only when grown in presence of xylan, the necessity of xylan as inducer is evident. The inducible nature of the two strains under study is in agreement with earlier reports on xylanases (Gascoigne and Gascoigne, 1960; King and Fuller, 1968; Howard et al., 1960). This fact cotrasts with some reports concerning β -D-xylanases in eukaryotic microorganisms. Notoria et al. (1976 and 1979) demonstrated the constitutive nature of these enzymes in Cryptococcus

albidus var aereus, enzyme is synthesized even in presence of glucose as sole carbon source.

The extracellular xylanase activity was obtained from culture filtrates of S.flavogriseus and S.wedmorensis grown on different substrates. Both S.wedmorensis and S.flavogriseus exhibit versatility in utilizing different carbon sources as inducers of xylanase. For S.flavogriseus xylan and lactose were found to be the most effective inducers, mannose and galactose also induced activity but to lower extent, on avicel as substrate, the enzyme production was minimal for first 48 hrs, then the activity increased during next 24 hrs to about 5 I.U. and levelled off thereafter, however, when xylan served as carbon source considerably higher xylanase activity was obtained reaching 45 I.U. in 48 hrs. Glycerol, sucrose, fructose, arabinose, xylose, cellobiose, maltose and glucose were found to be poor inducers. Mannose and galactose also induced xylanolytic activity which is logical as D-mannose and D-galacto pyranosyl residues are often found in varying amounts along with acetate and uronic acid residues in xylan (Timel, 1967).

In S.wedmorensis, xylan (1%) was recognised as best inducer thus showing clearly an inductive effect of xylan on its hydrolytic enzyme, other effective inducers included lactose, galactose and mannose whereas with D-arabinose, xylose, maltose and glucose activity was at basal level and it did not increase with time.

The fact that xylan is the best carbohydrate for β -D-xylanases induction is in agreement with the results published

by Nakanishi (1976) on β-D-xylanase synthesis by Streptomyces sp. The xylanases produced by these strains are comparable with those described in literature. Their pH values for maximum catalytic activity are described in the range of neutral or slightly lower, whereas for S.wedmorensis, maximum xylanase activity was exhibited at pH 7 and xylanase was stable at alkaline pH. The crude enzyme degraded xylan at pH 8 and pH 7 with almost parallel rate. High xylanase activity at pH 8 is of distinct advantage as maximum lignin loss occurs over a wide range of initial pH values (7.2 to 8.8) with actinomycetes. Also the alkaline xylanases are expected to attack xylan more readily since xylan is soluble in alkaline solution but insoluble in water. Alkaline xylanases from Bacillus sp. have been reported (Honda et al., 1985). In case of S.flavogriseus, the optimal pH for xylanase activity was 6, whereas in alkaline side there was heavy decline in activity. Stable xylanases with broad pH range should facilitate use of xylan as raw material.

The Streptomycete xylanases have relatively high thermostability like those of other bacteria such as Bacillus while the temperature optima is similar to that of fungal xylanases (Hagerdahl et al., 1978). S.wedmorensis xylanase has another stiking feature of stability at higher temperature. The enzyme was stable at 50^{o}C for more than 48 hrs retaining 75-79% activity. At 60^{o}C the crude enzyme had half life of 5 hrs which is much higher than reported in literature so far, whereas for S.flavogriseus at

40°C its half life is 24 hrs and this time was shortened considerably to 2.5 hrs at 50°C.

However, as reported for cellulolytic enzyme (Ishaque and Kluepfel, 1980), the xylanases of S.wedmorensis as well as S.flavogriseus are considerably more stable in presence of substrate. In S.flavogriseus, presence of substrate was found to extend the half life of enzyme from 2.5 to 7 hrs at 50°C. Similarly, at 40°C the half life was increased from 24 hrs to 40 hrs. In S.wedmorensis, at 60°C the half life was extended from 5 to 9 hrs.

Km value for S.flavogriseus was found to be 0.75 mg/ml which is comparable with S.lividans km 0.60 mg/ml, Km value is low compared to fungal and yeast xylanases which range from 4-20 mg of xylan per ml. S.wedmorensis has higher affinity for substrate Km is calculated to be 0.60 mg/ml.

All the divalent cations added to standard xylanase assay in S.wedmorensis and S.flavogriseus, substantial inhibition occurred with addition of 200 uM of Hg^{2+} whereas moderate inhibition occured with 200 um Ag^{2+}, other cations i.e. Cu^{+2} and Mg^{+2} only caused substantial inhibition when present in concentrations of 10 or 100 mM. The overall lack of inhibition of xylanase degrading activity by these metals except at very high concentrations indicates that metal precipitation technique could possibly be used to help purify this activity. The addition of 50 mM and 100 mM EDTA to the

assay resulted in only 5% and 12% inhibition in S.flavogriseus and 0% and 5% inhibition in S.wedmorensis suggesting that these compounds were not chelating cations required by xylanase.

The S.wedmorensis xylanase activity was characterized on polyacrylamide gel (Fig.2) distinct endoxylanase activity band was detected in crude enzyme preparation of S.wedmorensis in non-denaturing polyacrylamide gel, which indicates that xylan degrading activity of S.wedmorensis is true xylanase activity.

The activity was lost in boiling hence enzyme activity band could not be detected in SDS denaturing gel.

S.flavogriseus (Ishaque and Kluepfel, 1982) when grown on xylan induces both xylanase and cellulase enzymes. This seems to indicate that parts of xylanase gene exert some control on the expression of cellulase gene.

The product inhibition demonstrated by S.flavogriseus on adding xylose or glucose in medium containing xylan points towards a regulatory mechanism in which besides the necessity of inducer, xylose and glucose are also affecting the synthesis of β-D-xylanase. The question whether or not the effect is due to catabolic repression can be answered only by studying effect of cAMP in glucose growing strain.

These microorganisms can be suggested as a model for studying the synthesis and regulation of xylan hydrolytic enzyme when

using a variety of inducers and also for studying in a comparative way the difference and convergence between prokaryotic and eukoryatic microorganisms in relation to utilization of complex polysaccharides.

These strains can be a choice organisms for utilization of xylan in native form which streptomyces are known to utilize as they produce cellulases, hemicellulases and lignolytic enzymes (Crawfod, 1981; Kluepfel and Ishaque, 1982) which are all essential for the complete degradation of lignocellulose biomass. From our studies it appears that S.wedmorensis will be a better choice as xylanase from this strain in crude form was fairly stable at 50^{o}C and alkaline pH 8. Stable xylanases with broad pH range should facilitate the use of xylan as raw material, hence the strain should serve as a good candidate for gene manipulation.

With the advent of recombinant DNA technology it has been possible to isolate and clone the genes for specific enzymes in multicopy vector thereby giving a gene dosage effect. For this we ultimately need a gene coding for xylanase which is sturdy enough to bear the rigorous conditions of fermentor i.e. extremes of pH and temperature. For this purpose S.wedmorensis would particularly serve as good donor strain or manipulations could be made in strain itself as cloning vectors for streptomyces are available.

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